THE TYPE III SECRETION SYSTEM OF *Pseudomonas aeruginosa*: APPLICATIONS AND THE DISCOVERY OF A NOVEL CYTOTOXIN

By

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To my wife and grandmother, whose encouragement and support helped make me who I am today as a scientist

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ADPRT	ADP-Ribosyltransferase domain
BSA	Bovine serum albumin
CF	Cystic fibrosis
DMEM	Dulbecco's modified eagle media
DNA	Deoxyribonucleic acid
EA	Exotoxin A
E. coli	*Escherichia coli*
ExoS	Exoenzyme S
ExoT	Exoenzyme T
ExoU	Exoenzyme U
ExoY	Exoenzyme Y
FBS	Fetal bovine serum
GAP	GTPase activating protein
GFP	Green fluorescent protein
iPS cell	Induced pluripotent stem cell
kD	Kiladalton
L broth	Luria broth
MDM2	Murine double minute 2
MEF	Mouse embryonic fibroblast
MLD	Membrane localization domain
MOI	Multiplicity of infection
NDK	Nucleoside diphosphate kinase
NDP	Nucleotide diphosphate
NGS	Normal goat serum

NTP	Nucleotide triphosphate
NLS	Nuclear localization sequence
OD	Optical density
P. aeruginosa	*Pseudomonas aeruginosa*
PBS	Phosphate buffered saline
PI	Propidium iodide
PLA	Phospholipase A_2 activity
SOD	Superoxide dismutase
T1SS	Type I secretion system
T3SS	Type III secretion system
TCA	Trichloroacetic acid
X-Gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

Abstract of Dissertation Presented to the Graduate School
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THE TYPE III SECRETION SYSTEM OF *Pseudomonas aeruginosa*: APPLICATIONS AND THE DISCOVERY OF A NOVEL CYTOTOXIN

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Pseudomonas aeruginosa is an opportunistic pathogen responsible for causing diseases in immunocompromised individuals. Disease results from the production of numerous virulence factors, some of which are injected directly into the host via the type III secretion system (T3SS). Recent breakthroughs in the field of cell biology have demonstrated that eukaryotic cells could be transformed back into a pluripotent state by the ectopic expression of several transcription factors, however this is accomplished with the use of integrating oncogenic viral vectors. We therefore set out to develop a protein delivery system that could replace current methodologies, which would meet an emerging need in the field of cell biology. Studies have demonstrated that proteins of interest could be delivered into the cytosol of a host eukaryotic cell by fusion to the first 54 amino acids of the type III effector Exoenzyme S (ExoS). In this work, we demonstrate for the first time, the ability to deliver functional nuclear proteins into mammalian cells using a strain of *P. aeruginosa,* which is greatly diminished in cytotoxicity.

Although the protein delivery strain was reduced in cytotoxicity for incubations with cells for less than 3 hours, longer times still resulted in cytotoxicity. Work presented in

this study demonstrates that residual toxicity results from the injection of nucleoside diphosphate kinase (NDK) into host cells. Evidence presented in this work shows that NDK is not injected like traditional type III effectors, but instead is secreted first into the extracellular environment via a type I secretion system (T1SS) and then translocated into the host cell by the T3SS. These results are evidence in support of a newly emerging model of type III secretion whereby effectors are first secreted from the bacteria, and then injected into the target cell.

ExoS is a potent type III secreted toxin which has shown the ability to kill cancerous cells by inducing apoptosis. Since ExoS must be activated by eukaryotic 14-3-3 proteins, we examined if ExoS could be put under control of a cancer specific protein, murine double minute 2 (MDM2), by replacing the 14-3-3 binding domain with a domain for MDM2. Studies from this work show that while the manipulated form of ExoS was reduced in cytotoxicity, it still had a functional ADP-ribosylation domain, suggesting that MDM2 could play a role in the activation.

As a whole, the results presented in this work demonstrate that *P. aeruginosa* can be utilized as a protein delivery system. They also identified a novel type III injected effector, which is injected into host cells via a novel mechanism. Finally, work presented here suggests that ExoS has the potential to be manipulated in such a fashion as to promote cancer cell specific killing.

CHAPTER 1
INTRODUCTION

Pseudomonas aeruginosa

Basic Microbiology

Pseudomonas aeruginosa is the most studied member of the genus *Pseudomonas*, which belongs to the family Pseduomonadaceae. It is a ubiquitous gram-negative bacillus that is found throughout the environment, as well as in many areas of hospitals (38). *P. aeruginosa* is classified as an obligate aerobe and typically nonfermentative (163). The ability to survive in many different environments results from the fact that it can use over 30 different organic compounds for growth and can grow at higher temperatures than most other enteric organisms (116, 163).

Role as a Human Pathogen

Although *P. aeruginosa* is found throughout the environment, it functions as an opportunistic pathogen that typically causes diseases in immunocompromised individuals such as patients suffering from HIV or patients undergoing chemotherapy (158) (166). Infections from this organism are also commonly observed in individuals who have suffered severe burn wounds (129). Surveillance studies have shown that *P. aeruginosa* is responsible for 11-13% of all nosocomial infections when a microbiological isolate was identifiable, and that this percentage is higher in infections found in intensive care units (ICUs) (37). The types of infections common to *P. aeruginosa* include, but are not limited to urinary tract infections, soft tissue infections, pneumonia, and even ulcerative keratitis (149, 165).

While *P. aeruginosa* is able to cause numerous types of infections, it is well known for its ability to colonize the lungs of patients suffering from cystic fibrosis (CF) (72). CF

is a genetic disease cause by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), which disrupt normal ion flow in lung epithelial cells (172). This results in elevated levels of mucus, which prevents normal clearing of particles from the lungs (171). In 2004, it was reported that 57% of reported respiratory cultures from CF patients contained isolates of *P. aeruginosa*, which highlight the importance of this organism in disease (37). One study has shown that the CFTR plays a role in the uptake and clearance of *P. aeruginosa* by lung epithelial cells. Additionally, it was shown that mutations in the CFTR gene prevent bacterial uptake and can promote conditions for chronic bacterial colonization (127).

Antibiotic Resistance

One of the major reasons that *P. aeruginosa* infections are able to thrive in hospitals is due to the emergence of multidrug resistant strains. These strains can display resistance to multiple drugs such as ceftazidime, ciprofloxacin, imipenem, and tobramycin (118). Due to its low outer membrane permeability, *P. aeruginosa* is naturally resistant to most antibiotics (18). In addition, *P. aeruginosa* possesses multiple drug efflux pumps, which function to remove antibiotics from the bacterial cytosol (97). Genetic mutations, as well as acquisition of novel resistance mechanisms through gene transfer or plasmid uptake, also contribute to the drug resistance seen in *P. aerugionsa* (18).

As a result of the increasing emergence of resistant strains, new strategies for treating *P. aeruginosa* infections are being developed. One such method is to treat infections using multiple drugs simultaneously, although studies are currently still examining the efficacy of such attempts (159). New strategies also include developing molecules that can target virulence factors such as the T3SS (2). A recent study has

reported that utilization of a mouse monoclonal antibody against PcrV, a protein at the tip of the needle, in combination with antibiotics was able to reduce disease in a mouse model of pneumonia when compared to the use of antibiotics alone (146). Studies have also identified small molecule inhibitors of ExoS, however these have not been used in animal studies (9).

Pseudomonas aeruginosa Virulence Factors

Flagellum

P. aeruginosa possesses a single polar flagellum that is utilized for motility and aids in biofilm formation (21). Studies have shown that the flagellum is responsible for stimulating Toll-like receptor 5 located on the outer surface of airway epithelial cells, which results in the production of proinflammatory cytokines (1). The importance of the flagellum in virulence is highlighted by infections in several animal models, which show that strains lacking flagellum are greatly attenuated in toxicity (41, 112). Observations from patients who are chronically infected with *P. aeruginosa* show approximately 39% of strains isolated do not produce flagella (103, 164). It is believed that after the initial colonization of *P. aeruginosa*, the bacteria down regulates expression of the flagella in an attempt to avoid the host immune system (12, 174).

Quorum Sensing and Biofilms

Quorum sensing is a mechanism that bacteria utilize in order to communicate with each other (47). *P. aeruginosa* has two defined quorum sensing pathways, the *las* and *rhl* systems (125). These systems work by producing small diffusible molecules, known as autoinducers, which are spread to surrounding bacteria and are responsible for binding to transcriptional activators and inducing expression of various genes (133). Quorum sensing has been linked to the expression of toxins such as Exotoxin A and

elastase (51). Additionally, studies have shown that the secreted autoinducers from *P. aeruginosa* are able to generate an immune response in the host cells (145).

Biofilms are highly organized bacterial communities encased in a polysaccharide coat (63). *P. aeruginosa* is well know for its ability to form biofilms in surgical implants and the lungs of CF patients (37). This type of growth is problematic due to the fact the polysaccharide coat protects the bacteria from antibiotics and other environmental stresses, which make it more difficult to treat the infections.

Type I Secretion System

P. aeruginosa is known to possess several type I secretion systems (17). These secretion systems are the most basic of the six known systems consisting of outer and inner membrane proteins connected by an adaptor protein. These systems are also commonly referred to as ABC transporters due to the fact the inner membrane proteins are ATP-bind cassette proteins which provide energy for protein secretion (35). Substrates destined for secretion through this system possess a non-cleavable secretion signal located in the C-terminal portion of the protein (17). During secretion, the signal sequence interacts with a nucleotide-binding domain located in the inner membrane protein and undergoes a conformational change, which presumably generates the energy for protein secretion through the hydrolysis of ATP (17). *P. aeruginosa* utilizes these systems to secrete virulence factors such as alkaline proteases and NDK (101).

Other *P. aeruginosa* Secretion Systems

Six types of secretion systems have been identified in gram-negative bacteria known as the type I through type VI secretion systems (64). While each of these systems are unique, they share a common function of transporting proteins across the

bacterial membranes. *P. aeruginosa* is known to possess type I, II, III, V, and VI secretion systems (101). The type II system is fairly conserved among gram-negative bacteria and is responsible for secreting proteins into surrounding media (25). In *P. aeruginosa*, the type II system is responsible for secreting the potent Exotoxin A (135). Type V secreted proteins are referred to as autotransporters due to the fact they encode a domain which forms a β-barrel for their own export from the bacteria (17). Type VI secretion systems are a recently discovered system. While there is still much to be learned about these systems, it has been shown that the *P. aeruginosa* system secretes a protein known as HcpI, which is able to generate an immune response in CF patients (115). Although *P. aeruginosa* possess several secretion systems, all of which contribute to virulence, the most known of these systems is the type III secretion system.

Exotoxin A

Exotoxin A (EA) is considered to be one the most potent toxins secreted by *P. aeruginosa*. It is secreted via the type II secretion system, which is controlled by a quorum sensing mechanism (135). The toxicity of EA results from its ability to ADP-ribosylate the eukaryotic elongation factor 2 protein, which ultimately leads to infected cells undergoing apoptosis (44). *P. aeruginosa* secretes EA into the surrounding media, where it binds to the 2-macroglobulin receptor located on the surface of lung epithelial cells and is internalized (87). Increased levels of EA in bronchial secretions of CF patients correlate with severity of the disease demonstrating the role of this toxin in *P. aeruginosa* pathogenesis (94).

Nucleoside Diphosphate Kinase

Nucleoside diphosphate kinase is a protein that is responsible for converting nucleotide diphosphates (NDP) into nucleotide triphosphates (NTP) by transfer of a terminal phosphate group (147) In *P. aeruginosa*, NDK has been shown to be secreted via a type I secretion system, although the exact transporter has yet to be identified (80). Reports have shown that secreted NDK is cytotoxic to macrophages, presumably through a mechanism where NDK disrupts extracellular ATP concentrations which eventually causes macrophages to undergo apoptosis (181). It has been reported that not all strains secrete NDK, but only the mucoid producing strains (181).

Pseudomonas aeruginosa Type III Secretion System

Type III Secretion System

The type III secretion system (T3SS) is a proteinacious needle that protrudes from the bacterial surface and is capable of delivering toxins, known as effectors, directly into the cytosol of eukaryotic cells (55, 91). Many gram-negative species, such as *Pseudomonas*, *Yersinia*, and *Shigella,* utilize this system in order to establish infection in host cells (32, 107, 121). The repertoire of effector molecules secreted by bacteria possessing the T3SS varies greatly, with some causing the uptake of bacteria for intracellular survival, and others inducing apoptosis (27). This secretion system is not only limited to bacteria that cause diseases in mammals, as plant pathogens also utilize it for survival in a variety of plant species (96). Bacteria are believed to have acquired the T3SS though horizontal gene transfer based on evidence, which demonstrates that these systems are typically encoded in pathogenicity islands in the bacterial chromosome. Additionally, the regions encoding the T3SS can also be located on

plasmids and bacteria can possess multiple systems located in different areas of the bacterial genome (59).

Structural Features of the T3SS

The T3SS is a macromolecular structure composed of more than 20 different proteins (168). At the core of the system is the basal body, which spans both bacterial membranes as well periplasmic space. It is composed of two ring like structures, one situated in the bacterial inner membrane and the other in the outer membrane linked together by a hollow inner rod (113). Interestingly, the basal body of the T3SS is very similar in structure and function to the flagellar basal body suggesting that these two systems might be evolutionarily related (102, 160).

Upon an appropriate T3SS inducing stimulus, the basal body secretes proteins that make up the needle of the T3SS. In *P. aeruginosa* the needle is composed of the PscF protein, which generates needles that can range from 60-70nm in length and 6-7nm in width (73, 122). Once the needle has been formed, the T3SS begins to secrete proteins that form the translocation complex located at the tip of the needle. This complex is composed of the PcrV, PopB, and PopD proteins in *P. aeruginosa* and is necessary for the injection of proteins into eukaryotic cells (62). PopB and PopD alone have been shown to form pores in lipid vesicles suggesting that these two proteins are responsible for pore formation when the bacteria come in contact with the host cell (57). PcvR is believed to control the pore size by facilitating the interaction of PopB and PopD at the tip of the needle (57).

Once the T3SS is completely assembled, it begins to secrete effector molecules. The number of effectors, as well as their function, varies greatly among bacterial species (27, 148). However, despite their differences, all effectors are believed to

encode a signal sequence, which facilitates their secretion through the T3SS (154). It is not unusual for bacteria to express several effectors simultaneously, yet bacteria are able to secrete them in a particular order. Recent work in *Salmonella typhimurium* has suggested that a cytoplasmic sorting platform exists in which effectors are loaded prior to secretion. Based on the loading order of proteins and the use of specialized chaperones, the T3SS is able to recognize the order in which proteins should be exported (93).

Regulation of T3SS in *P. aeruginosa*

Although it is known that the contact with host cells and low extracellular calcium concentrations are able to induce the T3SS, the exact mechanism behind this is not completely resolved (62). However, there has been much research into the regulation of the T3SS and its products. In *P. aeruginosa*, all of the genes associated with the T3SS including structural components, chaperones, and secreted effectors, are all under control of the transcriptional activator ExsA, which is a member of the AraC family of activators (20). Transcription of T3SS related genes is closely tied with type III secretion. When the type III system is repressed, ExsA is bound to an anti-activator ExsD, which blocks ExsA mediated transcription (177). However, under type III inducing conditions, an anti-anti-activator binds ExsD and frees ExsA to allow expression of type III secretion components (177).

***P. aeruginosa* Type III Secreted Effectors**

P. aeruginosa possesses four known type III secreted effectors: Exoenzyme S (ExoS), Exoenzyme T (ExoT), Exoenzyme U (ExoU), and Exoenzyme Y (ExoY) (38). Compared to other type III containing bacteria such as Salmonella, this is a relatively low number (27). Although *P. aeruginosa* has four effectors, strains usually only contain

three. Sequencing of numerous strains of *P. aeruginosa* revealed that a vast majority contains ExoT and ExoY. However, it has been shown that only 28-42% of strains contain ExoU, and 58-72% of strains have ExoS, but none contain both ExoS and ExoU (42). Therefore strains either possess ExoS, ExoT, and ExoY or ExoU, ExoT, and ExoY. The type of cytotoxic response seen in infected cells usually depends on whether the strains contain ExoU or ExoS, as these two toxins are the most cytotoxic.

ExoU: ExoU is the largest type III secreted effector from *P. aeruginosa* comprised of 687 amino acids and possessing a molecular weight of approximately 74kD (43). It is regarded as one of the most potent toxins expressed by *P. aeruginosa* due to its phospholipase A_2 (PLA) activity, which elicits cytotoxicity through its ability to cleave fatty acids at the sn-2 position resulting in products which are stimulators of the inflammatory response (138, 144). In order for ExoU to generate a toxic effect, it must be activated by the eukaryotic co-factor superoxide dismutase (SOD), which interacts with specific amino acids located in the C-terminal end of ExoU and induces a conformational change (140) (14, 137). Interestingly, ExoU is ubiquitinated, although this does not appear to have a negative impact on the ability of the protein to induce toxicity (150). Recent evidence actually suggests that ubiquitination might play a role in the activation of ExoU (7).

ExoT: ExoT is a 53 kD bifunctional toxin that shares 76% amino acid sequence homology with ExoS. Like ExoS, it possesses both a N-terminal GTPase activating protein domain (GAP) and a C-terminal ADP-ribosyltransferase domain (ADPRT) (53, 82). The GAP domain of ExoT is responsible for causing rounding of the infected cell by disrupting the functions of Rho, Rac, and CDC42 (88). In addition, it has also been

implicated in blocking cytokinesis by targeting RhoA (142). Studies have shown that the GAP domain of ExoT plays an inhibitory role in lung epithelial cell wound healing, which probably is a result of the disruption of the cellular cytoskeleton and focal adhesions (54).

Similar to ExoS, the ADPRT domain of ExoT requires activation from eukaryotic 14-3-3 proteins (11). While both toxins share similar ADPRT domains, the substrates they target differ. ExoS is known to induce apoptosis by ADP-ribosylating Ras proteins however, ExoT does not, yet is still able to induce apoptosis although at a slower rate than that by ExoS (152). The ADPRT of ExoT has been shown to target both CrkI and CrkII proteins, which results in inhibition of phagocytosis and bacterial uptake by the infected cell (37). Although ExoT has the ability to induce toxicity on its own, ExoS and ExoU often mask its toxic effects.

ExoY: ExoY is the smallest of the four type III secreted effectors encoded by *P. aeruginosa* with a molecular weight of 42 kD. It has been identified as an adenylate cyclase, with homology to other adenylate cyclases from both *Bordetella pertussis* and *Bacillus anthracis* (176). ExoY is responsible for altering cyclic AMP levels in infected cells, which ultimately lead to rounding and prevention of bacterial uptake (34). Like the other type III secreted effectors of *P. aeruginosa*, ExoY needs to be activated by a eukaryotic co-factor however, the identity of the factor is currently unknown (176). Recent studies have shown that the adenylate cyclase activity of ExoY is able to mediate bleb-niche formations in infected cells, similar to ExoS, although the exact role this plays in pathogenesis is currently under investigation (75).

ExoS: ExoS is a 453 amino acid protein comprised of several different functional domains that contribute to the cytotoxicity of infected cells (Figure 1-1). Located in the N-terminus of ExoS is the signal sequence necessary for its secretion through the T3SS (10). Once injected into host cells, ExoS localizes to the cell membrane via a leucine rich membrane localization domain (MLD) located from amino acids 51-72 (183). Studies have demonstrated that the MLD is also responsible for targeting ExoS to endosomes, which then transport it to the perinuclear region of the cell where it can interact with additional substrates (184).

ExoS is a bifunctional toxin containing two cytotoxic domains, a N-terminal GAP domain, and a C-terminal ADPRT domain (56, 130). The GAP domain is responsible for targeting the small molecular weight GTP-binding proteins (GTPases) Rho, Rac, and CDC42, which ultimately leads to the rounding and lifting of infected cells (68, 151). Typically, GTPase proteins cycle between an inactive GDP bound form and an active GTP bound form, but the GAP domain of ExoS forces proteins to be in the inactive GDP bound state, thereby disrupting cell signaling (4).

Unlike the GAP domain, the ADPRT domain requires activation from a eukaryotic co-factor, identified as 14-3-3 proteins, in order to generate toxicity (48). These proteins play critical roles in cell signaling by facilitating activities such as apoptosis, protein trafficking, and cell division (114). The interaction of ExoS with 14-3-3 proteins is unique due to the fact it binds in the opposite orientation of most known 14-3-3 ligands (120). Additionally, ExoS does not rely on the use of phosphoserine for binding whereas most known 14-3-3 binding partners do (66, 175). While it is clear that ExoS must interact with 14-3-3 to activate the ADPRT domain, the exact mechanism of activation remains

elusive. Based on the activities of 14-3-3 proteins, it is believed that these proteins induce a conformational change in the ADPRT, thereby rendering it active.

Once activated, the ADPRT domain of ExoS ADP-ribosylates various cell signaling proteins such as ezrin, moesin, vimentin, Ral, and Ras proteins (29, 46, 104). Of the known target proteins, the interaction with Ras is the most extensively studied. Ras is a GTPase protein responsible for regulating critical cellular responses such as proliferation, differentiation, and apoptosis (141). ExoS is able to block catalyzed nucleotide exchange of Ras, which inactivates it and leads to the inhibition of the ERK survival pathway (52, 67).

ExoS mediated cell death is complicated and still not completely understood. It has been demonstrated that cells expressing a constitutively active form of Ras can be rescued from the effects of ExoS, suggesting that Ras inhibition is the major cause of ExoS related toxicity (76). However, it has also been shown that the JNK pathway is also responsible for toxicity, as expression of a dominant negative JNK in HeLa cells reduces ExoS mediated apoptosis (77). Since the ADPRT domain has so many substrates, it is possible that the overall toxicity from ExoS does not come from disruption of just one pathway, but a combination of multiple pathways. Recent studies have also shown that the ADPRT of ExoS is able to promote bacterial penetration through tight junctions into the intestinal tract and aid in survival inside eukaryotic cells, which highlight the complexity of ExoS induced toxicity in infected cells (8, 119).

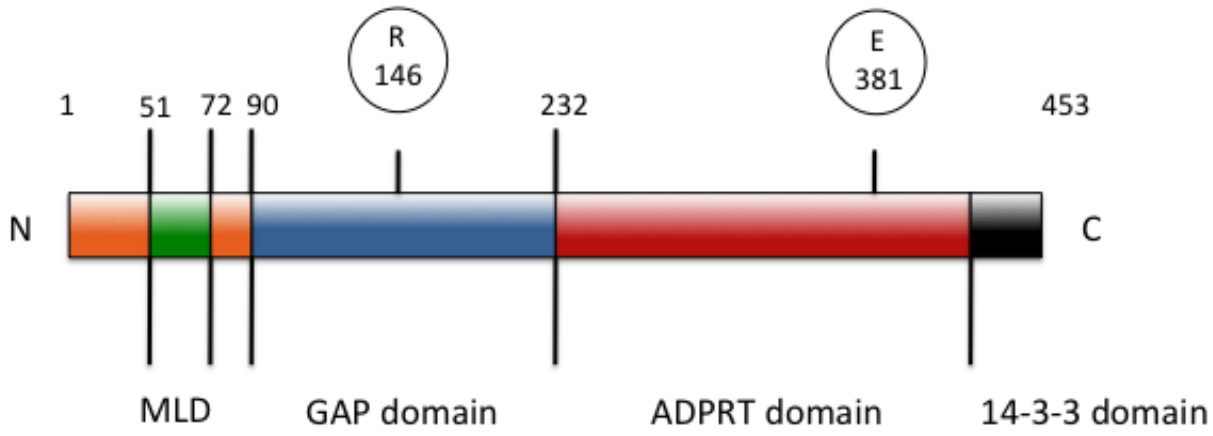

Figure 1-1. Diagram displaying the functional domains of ExoS. The green portion of ExoS represents the MLD domain. The blue segment corresponds to the GAP domain and the red portion is the ADPRT. Located in the back area is the C-terminal 14-3-3 binding domain. The amino acids circled are the catalytic sites for GAP and ADPRT activities.

CHAPTER 2
GENERAL MATERIALS AND METHODS

Bacterial Strains and Plasmids

The bacterial strains and plasmids used throughout this entire work are listed in Table 2-1 and Table 2-2 respectively. All plasmid constructs synthesized were designed using the primers listed in Table 2-3. Detailed information regarding the cloning of each construct is listed in each upcoming chapter. Both *E. coli* and *P. aeruginosa* were grown by shaking in Luria (L) broth or on L agar plates at 37° C. The final concentration of antibiotics used for plasmid selection was 100µg of carbenicillin per ml for *P. aeruginosa* and 100µg of ampicillin per ml for *E. coli.* Before both protein secretion and injection assays, *P. aeruginosa* strains were sub cultured by inoculating 100µl of an overnight culture into 2mL of fresh L broth containing antibiotics. For infection assays, bacteria were cultured to early log phase, which corresponds to an optical density (OD) of 0.8 to 1.0.

Eukaryotic Cell Culture

Both HeLa and mouse embryonic fibroblast (MEF) cell lines were grown in adherent monolayers by culturing them in Dulbecco's Modified Eagle Media (DMEM) (Gibco) with the addition of 5% fetal bovine serum (FBS). Te26 cells were cultured in DMEM containing 10% FBS, and both H1299 and H1299-HDM2 were grown in DMEM containing 15% FBS. All cell lines were supplemented with 100µg per ml of penicillin and 100µg per ml of streptomycin and were incubated at 37°C with 5% CO_2. H1299-HDM2 cells were also cultured with 500µg per ml of geneticin (G418) in order to select for the plasmid expressing the human form of the MDM2 protein. It should be noted that

all serum used was heat inactivated. This prevented bacteria from being killed by complement components present in the serum.

Bacterial Protein Secretion Assay

P. aeruginosa strains were grown overnight in a 37°C shaking incubator with L broth containing antibiotics. The following morning, bacteria were sub cultured and grown for 3 hours. Bacteria were then collected and pelleted by centrifugation at 20,000xg for 2 minutes. 100µl of supernatants were mixed with 100µl of protein sample loading buffer (BioRad). Samples were boiled for 10 minutes and then stored at -20°C until use. When indicated, supernatants were trichloroacetic acid (TCA) precipitated in order to improve visualization of protein on Western blots. This was accomplished by mixing the 1ml of bacterial supernatant with 200µl of TCA on ice for 30 minutes. Samples were then centrifuged at 20,000xg for 10 minutes to pellet down protein. The supernatants were removed and samples were washed in 250µl of acetone, followed by centrifugation at 20,000xg for 5 minutes. Supernatants were cleared, and the pellet was allowed to air dry at room temperature for 10 minutes. The remaining pellet was then suspended in 50µl of protein sample loading buffer and then subjected to boiling for 10 minutes.

Protein Injection Assay

Mammalian cells were seeded at 70% confluency (8.4×10^5 cells) in 6-well plates in DMEM containing antibiotics the night before infection. Two hours prior to infection, the media was removed and cells were washed 2 times in PBS to remove residual antibiotics, followed by addition of 1 ml of fresh DMEM lacking antibiotics. After changing the media, *P. aeruginosa* strains were grown until they reached early log phase. For an MOI 20, 2×10^7 CFU per ml of bacteria were incubated with the cells for

the desired length of time. Following infection, bacteria were cleared by removing media and washing the cells twice in PBS. Cells were then harvested by incubation with 0.25% trypsin for 5 minutes at 37°C and then pelleted by centrifugation at 500xg for 5 minutes. The supernatants were removed and the cells were washed in PBS 3 times in order to remove any bacteria still present. Cells were then subject to lysis by incubation with 0.25% Triton-X 100 in PBS for 10 minutes on ice, which only lyses mammalian cells. Following lysis, the cells were centrifuged at 20,000xg for 2 minutes and the supernatant was mixed with 50µl of protein sample loading buffer. Samples were then boiled for 10 minutes before storage at -20°C.

Table 2-1. List of bacterial strains used in this study

Strain	Description	Source of reference
P. aeruginosa		
PAO1	Common laboratory strain	(74)
PAK	Common laboratory strain	David Bradley
PAK-J	Derivative of PAK with enhanced T3SS	(16)
PAK-JΔ*pscF*	PAK-J derivative with deletion of *pscF*	This study
PAK-JΔ*popD*	PAK-J derivative with deletion of *popD*	This study
PAK-JΔ*exsA*	PAK-J derivative with deletion of *exsA*	This study
PAK-JΔ*S*	PAK-J derivative with deletion of *exoS*	This study
PAK-JΔ*T*	PAK-J derivative with deletion of *exoT*	This study
PAK-JΔ*ST*	PAK-J derivative with deletion of *exoS* and *exoT*	This study
PAK-JΔ*STY*	PAK-J derivative with deletion of *exoS, exoT,* and *exoY*	This study
PAK-JΔ*NSTY*	PAK-J derivative with deletion of *exoS, exoT, exoY,* and *popN*	This study
PAK-JΔ*7*	PAK-JΔ*NTSY* with deletion of *xcpQ, lasR,* and *lasI*	This study
PAK-JΔ*8*	PAK-JΔ*7* with deletion of *ndk*	This study
E. coli		
DH5α	F-$80%*lacZ*&M15 *endA1 recA1 hsdR17*(r-'m+') *supE44 thi-1 relA1 &*(*lacZYA-argF*) *U169 gyr*A96*deo*R (92)	(61)
S17	Strain expressing DNA mobilization genes	This study

Table 2-2. List of plasmids used in this study

Plasmid	Description	Source of reference
pUCP20	*Escherichia-Pseudomonas* shuttle vector Ap^r Cb^r	(170)
pUCP18	*Escherichia-Pseudomonas* shuttle vector Ap^r Cb^r	(170)
pEX18TC	Vector containing *sacB* and Tc^r for exconjugant selection	(70)
pEX18TC-Δ*S*	pEX18TC containing 1kb regions upstream and downstream of *exoS* Tc^r	This study
pEX18TC-Δ*T*	pEX18TC containing 1kb regions upstream and downstream of *exoT* Tc^r	This study
pEX18TC-Δ*Y*	pEX18TC containing 1kb regions upstream and downstream of *exoY* Tc^r	This study
pExoS17-Cre	17 aa of ExoS fused to cre-recombinase in pUCP20 Cb^r	(16)
pExoS54-Cre	54 aa of ExoS fused to cre-recombinase in pUCP20 Cb^r	(16)
pExoS96-Cre	96 aa of ExoS fused to cre-recombinase in pUCP20 Cb^r	(16)
pExoS234-Cre	234 aa of ExoS fused to cre-recombinase in pUCP20 Cb^r	(16)
pExoS-Cre	Full length ExoS fused to cre-recombinase in pUCP20 Cb^r	(16)
pHW0224	*exoS* mutated at R146K E381A in pUCP18 Cb^r	(60)
pHW0029	Wild-type *exoS* in pUCP18 Cb^r	(60)
pPaNDK	Flag tagged NDK from *P. aeruginosa* in pUCP20 Cb^r	This study
pPaNDKH117Q	pPaNDK with kinase null NDK Cb^r	This study
pEcNDK	Flag tagged NDK from *E. coli* in pUCP20 Cb^r	This study
pHuNDK	Flag tagged NDK of human origin in pUCP20 Cb^r	This study
pCDNA3.1(+)	Eukaryotic expression vector containing CMV promoter Ap^r	Invitrogen
pJJ0322	*exoS* in pEGFP-C1; Km^r	(78)
pDNNDK	*ndk* from *P. aeruginosa* in pCDNA3.1(+) Ap^r	This study
pDNNDKH117Q	pDNNDK with kinase null *ndk* mutant Ap^r	This study
pEGFP-1	Constitutive mammalian expression vector containing egfp (CMV promoter); Km^r	BD Clontech
pNDK133	133 aa of NDK with Flag tag in pUCP20 Cb^r	This study
pExoSP53	P53 binding domain in place of ExoS 14-3-3 binding domain in pUCP20 Cb^r	This study
pExoSPDIQ	High affinity P53 binding domain in place of ExoS 14-3-3 binding domain in pUCP20 Cb^r	This study

Table 2-2. Continued

Plasmid	Description	Source of reference
pExoS1E6N	P53 null binding domain in place ExoS 14-3-3 binding domain in pUCP20 Cb^r	This study
pExoSΔ14-3-3	Deletion of 14-3-3 binding domain of ExoS in pUCP20 Cb^r	This study
pExoSRK	ExoS containing catalytically inactive GAP domain	This study

Table 2-3. List of primers used in this study

Primer	Sequence: 5'-3'
ExoS-Cre Fusions	
ExoS-up	GACGAATTCGGCGTCTTCCGAGTCACTGGAGGC
ExoS17-dn	GACGAGTCGTGCAATTCGACGGCGAAAGACGG
ExoS54-dn	GAGCTCGAGCAGCCCCTCACCCTTCGGCGCGTCC
ExoS96-dn	GACGAGCTCGGACATCAGCGCAGGCTGCGCGTC
ExoS234-dn	GACGAGCTCCTTGTCGGCCGATACTCTGCTGAC
FullExoS-dn	GACGAGCTCGGCCAGATCAAGGCCGCGCATCCT
Cre-up	GGAGCTCATGCCTAAGAAGAAACGAAAGATC
Cre-dn	CGAGGTCGACGGTATCGATAAGCTTG
T3SS deletions	
ExoS-up	1. CAAGGAATTCGGATTATGCGGAGGGGTTGCCGGTG
	2. GTTGAGATCTCCTGATGTTTCTCCGCCAGTCTAGGAA
ExoS-dn	1. GTCCAGATCTTGGCTCGGCAGCGGATCCGGGTGGAG
	2. TGGAAAGCTTCGTCATCCTCAATCCGTACGGCAGGC
ExoT-up	1. GGAGGAATTCGAAGGGGTTGCGCAGGCCTGGCTCGTC
	2. TGACGGATCCTGATGTTTCCCCGCCAGTCTAGGAACG
ExoT-dn	1. CGGAGGATCCCAAGGGGTGTCCGTTTTCATTTGCGCC
	2. AGGTAAGCTTCCAGCGCCTGCGCCTGGGCCTCCTTG
ExoY-up	1. AACTGAATTCCGAGGATGTCGCCCTGCTCGACCATCGG
	2. CCCAGGATCCAGGAGGCGCTCGACTTTTTCCAACGTA
ExoY-dn	1. ATAAGGATCCGGGCAGCGGCGAGATATCAGAAAACG
	2. CGTTAAGCTTGAGATAGCCGAGCATGCTCAGGCCGTC
NDK constucts	
PaNDK-up	GGAGAATTCGCGCCTGGCCATCGCGGCGCAGATGG
PaNDK-dn	GGACTGCAGTCACTTGTCGTCATCGTCCTTGTAGTCGCGAA TGCGCTCGCAGACTTCGGTAGCCGC
EcNDK-up	ACCGGATCCCGCGACAGTGAAATTTGTCATGCAATAGTC
EcNDK-dn	ACCAAGCTTTCACTTGTCGTCATCGTCCTTGTAGTCACGGGT GCGCGGGCACACTTCGCCTTC
HuNDK-up	ACCGGATCCCGCGACAGTGAAATTTGTCATGCAATAGTC
HuNDK-dn	ACCAAGCTTTCACTTGTCGTCATCGTCCTTGTAGTCTTCATA GACCCAGTCATGAGCACAAGA
PaNDKpCDNA-up	ACCGGATCCGCCATGGCACTGCAACGCACCCTGTCCATCAT C
PaNDKpCDNA-dn	ACCGAATTCTCAGCGAATGCGCTCGCAGACTTCGGTAGCCG C
NDK133-dn	GGAGAGCTCTCACTTGTCGTCATCGTCCTTGTAGTCGAAGA AGTAGGCGATCTCGCGAGCGG
H117Q mutations	
NDKH117Q-up	CGAGAACGCCGTCCAGGGATCCGATTCCGAAGCTTCC
NDKH117Q-dn	GGAAGCTTCGGAATCGGATCCCTGGACGGCGTTCTCG

Table 2-3. Continued

Primer	Sequence: 5'-3'
ExoS-P53 Fusions	
F-ExoS-R1	CAGGAATTCGAGTTGATGGTGGATCTGGGCCC
ExoSP53-dn	AGCAAGCTTTCAGTTCTCGGGCAGATGCTTCCACAGGTCGC TGAAGGTTTCCTGGCTATGGCCACTCTGCTCCCCCAG
ExoSPDIQ-dn	AGCAAGCTTTCAGTTGCTCAGAAGCTGTGACCACCAATGTT CGAAGGTTTCCTGGCTATGGCCACTCTGCTCCCCCAG
ExoS1E6N-dn	AGCAAGCTTTCAGTTGCTGGTCAGCTGGGCCCAGTTATGTT CGAAGGTTTCCTGGCTATGGCCACTCTGCTCCCCCAG
ExoSΔ14-3-3-dn	AGCAAGCTTTCACTGGCTATGGCCACTCTGCTCCCCCAG

CHAPTER 3
BACTERIAL DELIVERY OF FUNCTIONAL NUCLEAR PROTEINS INTO DIFFERENTIATED CELLS

Background

Bacterial pathogens are equipped with a plethora of virulence factors that enable them to modify eukaryotic cells and cause diseases in humans. One common factor utilized by many gram-negative pathogens is the type III secretion system (T3SS). The T3SS is a proteinacious needle that is used to deliver cytotoxins, known as effectors, directly from the bacteria, into the cytosol of a host eukaryotic cell (31, 50). While this system is highly conserved among bacteria, the secreted effectors can display diverse functions in eukaryotic cells ranging from the induction of apoptosis to bacterial uptake (27, 148). Although the effectors can differ greatly in function, they all contain a variable N-terminal signal sequence that is responsible for guiding them through the T3SS (99, 154).

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that is responsible for causing diseases in immunocompromised individuals, most notably those suffering from cystic fibrosis or severe burn wounds (100). Disease results from production of a number of virulence factors, but most prominently from the T3SS. The T3SS of *P. aeruginosa* is highly regulated, both at the transcriptional and translational levels, and becomes activated during the course of infection when the bacteria come in contact with host cells, although the exact mechanism is not well characterized (177). *P. aeruginosa* contains four known type III secreted effectors, although strains only harbor three of them (62). Exoenzyme Y (ExoY) is an adenylate cyclase and Exoenzyme U (ExoU) is a potent phospholipase that breaks down eukaryotic cell walls. The remaining effectors, Exoenzyme S (ExoS) and Exoenzyme T (ExoT), are highly homologous bi-

functional toxins that possess ADP-ribosyltransferase activities (ADPRT) as well as GTPase activating protein (GAP) domains (33). Of these effectors, ExoS is the most studied and best characterized. Recent studies have demonstrated that functional proteins could be delivered into the cytosol of eukaryotic cells by fusing them to various lengths of the N-terminus of ExoS (39, 128). Specifically, ovalbumin fused to the first 54 amino acids of Exos was shown to be injected into the cells of a mouse, thereby inducing a $CD8^+$ T-lymphocyte response against the infected cells (40).

Recent breakthroughs in the field of cell biology have demonstrated that terminally differentiated cells could be reprogrammed back into a pluripotent state by forced exogenous expression of four transcription factors: Oct4, Sox2, cMyc, and Klf4 (153),(180). While this technology holds tremendous potential for clinical therapies, there are some reservations. Current protocols for deriving induced pluripotent stem cells (iPS cells) rely on the use of integrating oncogenic viral vectors for transgene expression. Additional methods to overcome this such as DNA transfection and the use of purified proteins have had some success, although the efficiencies of reprogramming are much lower (143, 179, 186). Development of a simple and efficient protein delivery system would satisfy an emerging need in the field of cell biology. While it has been demonstrated that *P. aeruginosa* can be manipulated to deliver proteins of interest into the cytosol of eukaryotic cells, one has yet to demonstrate successful delivery of nuclear proteins.

Cre-recombinase is a widely used genetic tool in the field of molecular biology, especially for the purpose of generating conditional gene knockouts in animal models (108). It is a bacteriophage derived protein which excises DNA sequences that are

flanked by loxP sites. For proper execution of its recombinase activity, Cre must localize to the nucleus in order to come in contact with target DNA, making it a convenient assay system to demonstrate the delivery of nuclear proteins (139).

In this study, we are the first to use *P. aeruginosa* to successfully deliver a functional nuclear protein into eukaryotic cells. We demonstrate that Cre-recombinase fused to the first 54 amino acids of ExoS is sufficient for delivery into host cells, and that this fusion protein is functional. Results presented here also show that amount of protein delivered can be adjusted by manipulating either the duration of infection or the multiplicity of infection (MOI). Our results also suggest that the cell cycle plays a role in the recombination efficiency. Taken together, results presented here demonstrate the *P. aeruginosa* is capable of delivering functional nuclear proteins into host cells thereby paving the way for future studies using this system to generate iPS cells.

Materials and Methods

Generation of ExoS-Cre Fusion Proteins

The ExoS-Cre fusion proteins were generated by PCR amplification of various 5' lengths of ExoS using the primers listed in Table 2-3. Each primer contained either an *EcoRI* or *SalI* restriction site for convenient cloning into the pUCP20 vector. Cre-recombinase was PCR amplified to include an SV40 large T antigen nuclear localization sequence at its 3' end using primers listed in Table 2-3. The variable portion of ExoS and Cre-recombinase were subject to a triple ligation with the *P. aeruginosa* vector, pUCP20, using *EcoRI*, *SalI*, and *SacI* restriction sites. Upon completion, constructs were confirmed using restriction enzyme digestions as well as DNA sequencing.

Bacterial Expression of ExoS-Cre Fusion Proteins

ExoS-Cre fusion proteins were electroporated into the *P. aeruginosa* strain PAK-JΔ*STY*. The strains were then grown in L-broth containing 150μg per ml of carbenicillin and 5mM EGTA for 3 hours in a 37°C shaking incubator. Bacteria were collected and pelleted by centrifugation at 20,000xg for 2 minutes. The supernatants were removed and bacteria were lysed by adding 150μl of protein loading buffer and boiling for 10 minutes. Bacterial lysates were then run on a 4-20% gradient SDS-PAGE gel and subjected to Western blotting with an antibody against Cre-recombinase (Abcam ab41104).

Bacterial Protein Secretion Assay

The bacteria strains PA01, PAK, and PAK-J were grown in a shaker at 37°C in L-broth containing 5mM EGTA. Bacteria were collected and pelleted by centrifugation for two minutes at 20,000xg. 100μl of bacterial supernatants were mixed with 100μl of protein sample buffer and boiled for 10 minutes. Samples were run on SDS-PAGE gels and subjected to Western blotting with an anti-ExoS antibody (generated from rabbits by Lampire Biological Laboratories INC), which is capable of detecting both ExoS and ExoT.

Protein Injection Assay

Te26 cells were seeded into 6 well plates at approximately 70% confluency (8.4×10^5 cells) in medium containing antibiotic the night before infection. Two hours prior to infection, cells were washed twice in 1X PBS and replaced with medium containing no antibiotics. Bacterial strains were grown in L broth supplemented with carbenicillin at 37˚C until the OD_{600} reached 0.8. For an MOI of 50, 5×10^7 CFU per ml of bacteria were incubated with Te26 cells for the indicated amount of time. Following infection, bacteria

were washed away and cells were harvested by scraping. Cells were spun down at 500xg for 5 minutes and then washed with PBS for 3 times. After centrifugation, Te26 cell pellets were suspended in 100μl of protein loading buffer. Samples were boiled for 10 minutes and subjected to Western blotting using an antibody against Cre-recombinase.

Immunostaining

MEF cells were infected with the *P. aeruginosa* strain PAK for 3 hours at an MOI of 20. Bacteria were cleared by removing the infection media and washing the cells 3 times in 1X PBS, and then cells were fixed with 3.7% formaldehyde in PBS at room temperature for 15 minutes. The cells were then washed 3 times with PBS and permeablized with 0.5% Trion X-100 in PBS. Cells were washed 3 times with PBS and blocked with 1% BSA in PBST for 30 minutes followed by incubation in the primary antibody for 2 hours at room temperature (α-ExoS 1:400). The cells were then incubated in the secondary for 1 hour at room temperature (α-Rabbit conjugated to fluorophore 488) and then washed 3 times in PBST before visualizing under a fluorescence microscope.

Cytotoxicity Assay

MEF cells were infected with either PAK-J or PAK-JΔ*STY* for 3 hours at MOIs 20, 100, and 500. Following infection, dead cells were removed by washing cells 3 times with PBS. The remaining cells were then collected by incubation with 0.25% trypsin for 5 minutes at 37°C. The number of viable cells was then counted under a microscope using a hemocytometer.

β-galactosidase Staining of Te26 Cells

Te26 cells were infected with PAK-JΔ*STY*/pExoS54-Cre at various times and MOIs as listed above. Following infection, the media was removed and cells were washed twice with PBS to remove residual bacteria. The cells were then fixed for 5 minutes at room temperature in a 1% formaldehyde and 0.2% glutaraldehyde in PBS solution. The cells were then washed 3 times and strained for β-galactosidase with a solution containing 4mM $K_4Fe(CN)_6$, 4mM $K_3Fe(CN)_6$, 2mM $MgCl_2$, and 0.4 mg per ml of X-gal at 37°C for 16 hours. The cells were then rinsed two times with water and the number of blue cells was counted under the microscope.

Cell Synchronization Protocol

Cells were synchronized to the early S-phase using a double thymidine block. Te26 cells were seeded at 25% confluency (3×10^5 cells) in DMEM containing antibiotics the night before synchronization. The next morning, cells were washed 2 times with PBS and fresh media containing antibiotics and 2mM thymidine was added for 18 hours. Afterwards, cells were released from the first thymidine block by washing twice in PBS and adding fresh media containing antibiotics for 9 hours. Cells were then washed once with PBS and the second thymidine block was started by adding media containing antibiotics and 2mM thymidine for 17 hours. Cells were then released from the second thymidine block by washing the cells in PBS and adding fresh media containing antibiotics. For infections assays, cells were released from the second thymidine block into media lacking antibiotics. After the second block, cells are synchronized to the early S-phase and then proceed into M-phase and the rest of the cell cycle.

Flow Cytometry Determining Phases of Cell Cycle

Te26 cells were double blocked in thymidine as described above. Following the block, cells were collected at various time points in order to determine a time frame for each phase of the cell cycle. DNA content was determined by propidium iodide (PI) staining, followed by flow cytometry of the cells. Te26 cells were collected by incubating cells for 5 minutes at 37°C with 0.25% trypsin. Cells were then fixed overnight at 4°C in 70% ethanol. After fixation, cells were pelleted by centrifugation at 500xg for 5 minutes and stained with 500µl of a PI staining solution (0.1% BSA, 0.1% RNase, and 0.5 mg per ml of PI) for 30 minutes at room temperature in the dark. Cells were then subject to flow cytometry to determine the content of DNA.

Results

Generation of a *P. aeruginosa* Strain for Protein Delivery

The common laboratory strain of *P. aeruginosa* (PAO1) secretes relatively low amounts of the type III effectors under type III inducing conditions. Therefore, we set out to identify a strain that was elevated for type III secretion with the goal of using this as the strain for protein delivery into eukaryotic cells. Various laboratory, as well as clinical and environmental strains, were tested for their ability to secrete type III effectors by collecting supernatants after the bacteria were cultured under type III inducing conditions for three hours. Supernatants were then run on SDS-page gel and subjected to Western blotting with an antibody against ExoS, which detects both ExoS and ExoT due to their high sequence homology (11). Figure 3-1 shows that another laboratory strain, PAK, secretes higher levels of type III effectors than PA01. Interestingly, an isolate of PAK that has been passaged in our lab for over 10 years (referred to as PAK-J) displayed even higher secretion of ExoS and ExoT. Quantitative ELISA assays from

our laboratory have demonstrated that PAK-J secretes more than 10 times the amount of ExoS as the standard PAK strain (84). However, the exact mechanism explaining this is currently under investigation. As a result of its elevated secretion, PAK-J is able to cause significant cytotoxicity when cultured with mammalian cells primarily as a result of ExoS. ExoS is efficiently injected in nearly 100% of the cells it comes in contact with and localizes to the perinuclear and outer membranes, as can be seen in Figure 3-2 (89).

PAK-J possesses ExoS, ExoT, and ExoY, which account for most of the cytotoxicity associated with this strain. In order to have an efficient strain for protein delivery, it needs to be capable of prolonged incubations with mammalian cells. To accomplish this, all three secreted effectors were deleted by successive allelic exchange resulting in the *P. aeruginosa* strain known as PAK-JΔ*STY*. Infection of MEF cells with PAK-J at a MOI of 20 for three hours resulted in a 75% decrease in cell viability as compared to non-infected control cells (Figure 3-3). Toxicity was further increased by infection with higher MOIs of PAK-J as there was a 95% decrease in viability in cells incubated with this strain at an MOI of 500. Conversely, infection with PAK-JΔ*STY* for three hours at an MOI of 20 resulted in only a 25% decrease in cell viability, and increasing the MOI did not result in increased cytotoxicity (Figure 3-3). Therefore, based on these assays, we selected PAK-JΔ*STY* as the strain to use for protein delivery.

Injection of Cre Recombinase Through the Bacterial Type III Secretion System

Cre-recombinase is a bacteriophage-derived protein that is frequently used for excising specific DNA sequences that are flanked by LoxP sites (117). The Cre-Lox system was chosen because Cre is a DNA interacting protein that must migrate to the

nucleus in order to carry out its recombinase activity. Additionally, this assay system is convenient because there are many reporter cell lines available.

In order to determine the optimal signal sequence necessary for maximum injection of Cre-recombinase, we generated a series of fusion proteins consisting of various N-terminal lengths of ExoS fused to Cre, with an in-frame nuclear localization sequence at the fusion junction (Figure 3-4). These constructs were then introduced into the PAK-JΔ*STY* strain and assayed for protein expression by growing the bacteria under type III inducing conditions for three hours. Bacteria were then collected, lysed, and subject to Western blotting with an antibody against cre-recombinase. As seen in Figure 3-5, all of the fusion proteins were synthesized at similar amounts with the exception of the full length ExoS fusion, which showed much lower production. To test for protein injection, a human sarcoma cell line (Te26) was infected with the various strains for three hours at an MOI of 100. Figure 3-6 shows that while the first 17 amino acids of ExoS appeared sufficient for injection of Cre, the first 54 amino acids facilitated an increase in protein injection. However, addition of more than 54 amino acids resulted in reduced protein translocation. To demonstrate that ExoS54-Cre injection was dependent on the T3SS, we infected Te26 cells with a type III defective strain (PAK-JΔ*popD*) expressing this construct, and observed no protein injection (Figure 3-6). Taken together, these results prove that ExoS54-Cre injection into mammalian cells is type III secretion dependent.

Functional Analysis of Bacterially Delivered Cre-Recombinase

In order to determine if bacterial delivered Cre-recombinase was functional, we utilized the Te26 cell line which contains an SV40 terminator flanked by LoxP sites preventing downstream expression of *lacZ* (Figure 3-7A). If ExoS54-Cre was functional,

the SV40 terminator would be excised allowing for *lacZ* expression, which can be detected with β-galactosidase staining. Te26 cells were infected with PAK-JΔ*STY*/pExoS54-Cre for 1-3 hours at various MOIs (20,100,500). Bacteria were then cleared and cells were allowed 48 hours to undergo recombination for *lacZ* expression, and then were stained with a solution containing 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-Gal) to assess β-galactosidase activity. As illustrated in Figure 3-7B, infection with PAK-JΔ*STY*/pExoS54-Cre results in blue cells as a result of *lacZ* expression due to removal of the SV40 terminator. β-galactosidase activity is mainly observed in the nucleus, which corresponds to the fact that fusion protein possesses a nuclear localization sequence (Figure 3-7B panel 4). The percentage of β-galactosidase positive cells increases in a dose dependent manner, as increasing either the duration of infection or MOI results in an increase in the number of blue cells (Figure 3-8). Examining the amount of protein injected by Western blotting indicates that the increase in blue cell correlates with increased protein injection (Figure 3-9). We also compared the amount of bacterial delivered Cre-recombinase to the amount produced by a lenti-virus that had integrated into the Te26 cell chromosome. Although the lenti-virus infected cells result in nearly 100% of the cells staining positive for β-galactosidase (Figure 3-7B panel 2), the amount of protein detected in those cells by Western blotting was undetectable (Figure 3-9). This is in contrast to the bacterial system, which is able to regulate the amount of protein injected by altering either the length of infection or MOI.

Cell Cycle Influences Recombination by Bacterially Delivered Cre-Recombinase

Immunofluorescent images above show that *P. aeruginosa* injects ExoS in nearly 100% of the cells it comes in contact with (Figure 3-2). However, infection with an MOI

of 500 for three hours results in only about 42% of the cells staining positive for β-galactosidase (Figure 3-8). Additionally, the amount of injected protein at that MOI and time is much higher than that produced from lenti-viral infected cells, yet viral infection results in nearly 100% of cells expressing β-galactosidase. It is plausible that in order for Cre to exert its recombinase activity, the chromosome needs to be in a relaxed or unwound conformation much like the chromosomes are during the S-phase of the cell cycle. In order to examine this, Te26 cells were subject to a double-thymidine block in order to order to synchronize cells to the G_1/S phase. Upon release from the thymidine block, cells were collected at various time points and subjected to flow cytometry in order to determine the percentage of cells in each phase of the cell cycle and to gain an overall time for Te26 cells to go through the entire cell cycle (Figures 3-10, 3-11). Infection with PAK-JΔ*STY*/pExoS54-Cre at an MOI of 500 for three hours in unsynchronized Te26 cells results in approximately 45% of cells staining positive for β-galactosidase, which is proportional to the number of cells in S-phase (Figures 3-8, 3-12). When Te26 cells are synchronized and infected during S-phase, the number of β-galactosidase positive cells increases to approximately 75%, which again, corresponds to almost exactly the number of cells in S-phase (Figure 3-12). To verify this phenomenon was dependent on the chromosome structure and not differences in injected protein, Te26 were infected during various phases of the cell cycle (G_1, S and G_2/M). The bacteria were cleared and cells were collected, lysed, and subject to Western blotting. Figure 3-13 indicates that the amount of ExoS54-Cre injected during the G_1 and S-phases was similar, suggesting the difference in β-galactosidase positive cells is not due to the amount of injected protein. Interestingly, the amount of protein

injected during the G_2/M phase was lower. It is possible that the amount is lower because the protein is either degraded or lost during the cell division process that occurs during that time. Taken together, these data suggest that the chromosome structure plays a role in the efficiency of Cre-mediated recombination.

Discussion

The bacterial T3SS has the capacity to replace current methodologies being used for cellular reprogramming. Current protocols rely on the use of oncogenic viral vectors, which become integrated into random areas of the host cell's genome, resulting in reservations about the use in clinical applications. Methods to bypass this, such as the use of purified proteins and transfection with plasmids, have yielded little success due to their extremely low efficiencies of reprogramming (143, 179, 186). In addition, these methods are very laborious and often times difficult to reproduce.

In this study we demonstrate for the first time, the ability to use the T3SS of *P. aeruginosa* to deliver a functional nuclear protein into eukaryotic cells. After screening numerous strains in our possession for secretion of type III effectors, we identified a strain, known as PAK-J, which secretes more than 10 times the amount of protein as the typical laboratory strain of PAK (Figure 3-1) (84). Normally, incubation of cells with this strain results in significant toxicity resulting from the secretion of type III effectors. However, we were able to reduce cytotoxicity dramatically by deletion of these proteins, thereby increasing the amount of time the bacteria could be incubated with host cells (Figure 3-3).

To demonstrate the ability to deliver nuclear proteins into eukaryotic cells, we employed the use of the Cre-LoxP system. Fusion of Cre-recombinase to various lengths of ExoS revealed that the N-terminal 54 amino acids was sufficient for maximum

delivery into host cells (Figures 3-4, 3-6). Although majority of the fusion constructs were produced at similar levels by *P. aeruginosa* (Figure 3-5), they did not have the same levels of injection. Our results show that while less than 54 amino acids could deliver Cre, more than 54 amino acids reduced the amount of injection. One possible explanation for this is that the fusions proteins containing more than the first 54 amino acids were too large to be exported through the type III apparatus. While the molecular weight of the largest construct (FullExoS-Cre) is approximately 80 kD, the largest protein secreted by *P. aeruginosa* is ExoU, having a molecular mass of 74 kD (43). However, PAK-J does not contain ExoU, but instead ExoS, which is the largest type III secreted protein by this strain having a mass of approximately 48 kD (10). Therefore it is reasonable to suggest that PAK-J might have difficulty in secreting proteins larger than 50 kD due to the lack of appropriate chaperone proteins.

To demonstrate functionality of the injected ExoS54-Cre fusion protein, we employed the use of a Te26 reporter cell line that contains a transcriptional terminator flanked by loxP sites, which inhibits *lacZ* expression. Infection with PAK-JΔ*STY*/pExoS54-Cre at an MOI of 50 for three hours resulted in the appearance of β-galactosidase positive cells (Figure 3-7B). The percentage of positive cells was dose dependent, as increasing either the MOI of duration of infection resulted in increased amounts of blue cells (Figure 3-8). This increase in β-galactosidase positive cells was accompanied by increased amounts of ExoS54-Cre injection, suggesting that greater amounts of protein result in a higher percentage of cells undergoing Cre-mediated recombination (Figure 3-9).

Interestingly, the maximum percentage of β-galactosidase positive cells we were able to obtain with bacterial infection was 45%, however infection of cells with a lenti-virus expressing Cre was able to achieve nearly 100%. Comparing the amount of protein produced by the lenti-virus infected cells, to the quantity delivered by bacteria, revealed that the bacteria were able to deliver a significantly higher amount (Figure 3-9). We reasoned that this discrepancy could be due to the fact that the lenti-virus is continually expressing a low level of protein, whereas the bacterial system is delivering a concentrated amount that will eventually degrade over time. In an effort to increase the percentage of cells undergoing recombination, we examined whether the phases of the cell cycle have any impact on the recombination efficiency. Since Cre-recombinase must interact with DNA to exert its activity, perhaps having the DNA in a more relaxed state, such as during the S-phase of the cell cycle, would increase the percentage of cells expressing β-galactosidase. This was indeed the case, as infecting Te26 cells synchronized to the S-phase of the cell cycle with bacteria increased the percentage of β-galactosidase positive cells to 75% as opposed to the 45% seen in unsynchronized cells (Figure 3-12). The percentage of cells expressing β-galactosidase after infection in both synchronized and unsynchronized cells corresponded directly to the number of cells that were in S-phase (Figure 3-12). This result was not dependent on the amount of injected protein during each phase, as Western blotting indicated the similar amounts were injected in both the S and G_1 phases (Figure 3-13). The G_2/M phase however, did show lower amounts of injected protein, probably resulting from the fact that cells are dividing and could potentially lose or breakdown the fusion protein.

The results presented here clearly demonstrate a proof of concept for the use of bacteria as a protein delivery system. Evidence presented here shows that we were able to achieve a 75% recombination efficiency using the bacterial system. It might be possible to even increase this percentage by treating cells with proteasome inhibitors or even subjecting cells to multiple rounds of infection. Although use of the lenti-virus is able to achieve nearly 100% recombination efficiency, the virus still inserts the gene of interest into the host cell chromosome. Using a bacterial system has the advantage in that it can be eliminated with the use of antibiotics, and the protein delivered is transiently expressed. The strain developed here is dramatically reduced in cytotoxicity thereby allowing it to be incubated with minimal harmful effects. The data presented in this study is proof that bacteria can be utilized to deliver functional nuclear proteins, paving the way for future studies using this system.

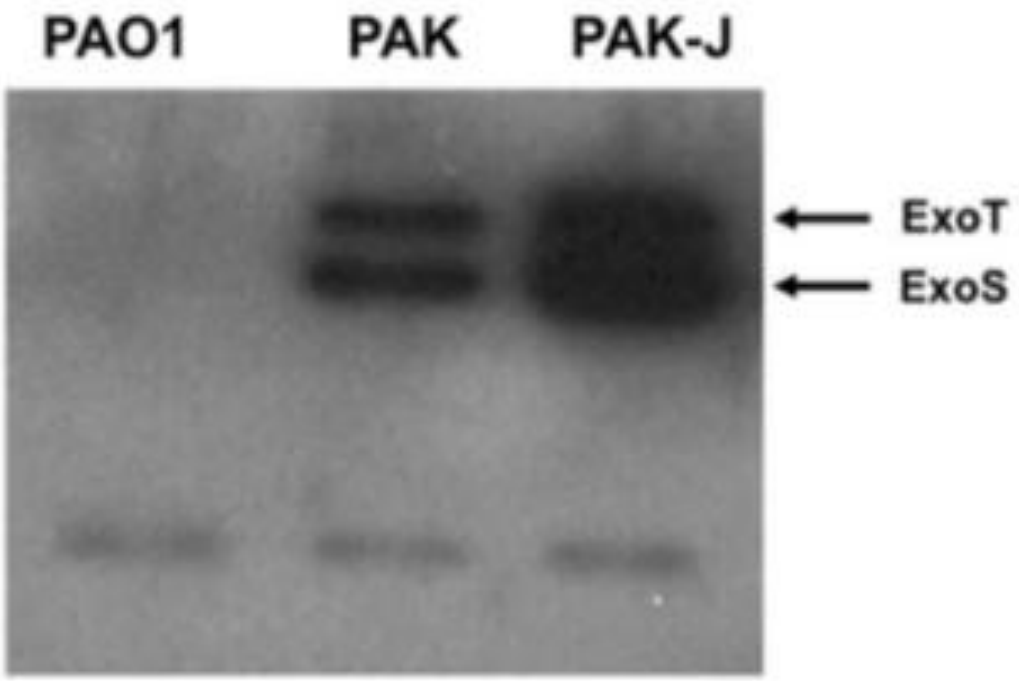

Figure 3-1. Type III secretion profiles of three laboratory strains of *P. aeruginosa*. Strains were grown under type III inducing conditions for 3 hours at 37°C. Bacteria were collected and then pelleted by centrifugation. Supernatants were collected and mixed with equal volumes of protein loading buffer and subject to Western blotting with an ExoS antibody which detects both ExoS and ExoT.

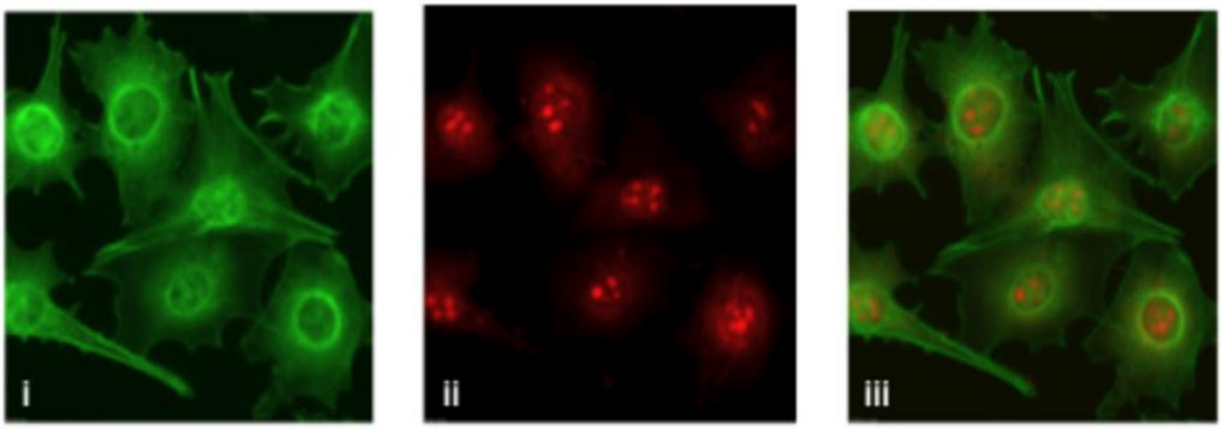

Figure 3-2. ExoS injection into HeLa cells via the T3SS of *P. aeruginosa*. MEF cells were infected with PAK-J for one hour at an MOI of 20.Cells were then fixed and stained with an ExoS antibody followed by incubation with a FITC labeled secondary antibody. i.) visualization of injected ExoS protein ii.) staining of nuclei with a propidium iodide stain iii.) overlay of injected ExoS and stained nuclei.

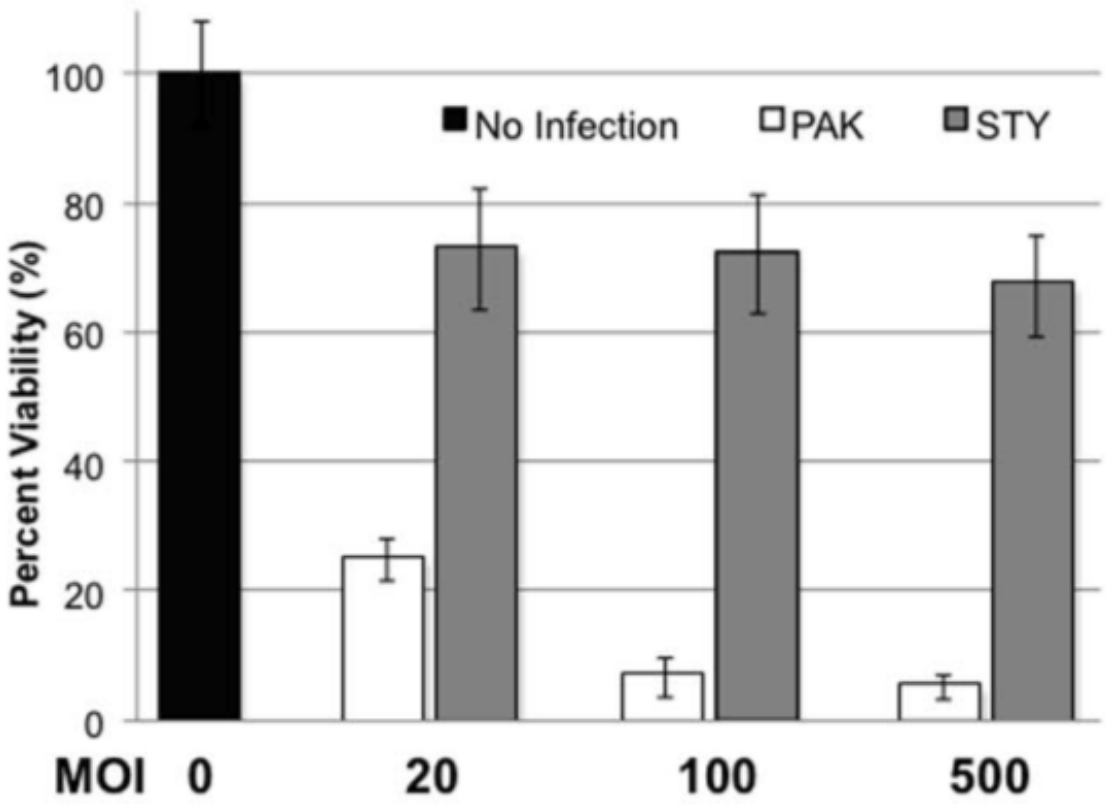

Figure 3-3. Viability of MEF cells following infection with *P. aeruginosa* strains. MEF cells were infected with the indicated strain of *P*. aeruginosa for 3 hours at the indicated MOI. Floating cells were removed and remaining adhered cells were trypsinized and counted using a hemocytometer. Data normalized to a non-infected control.

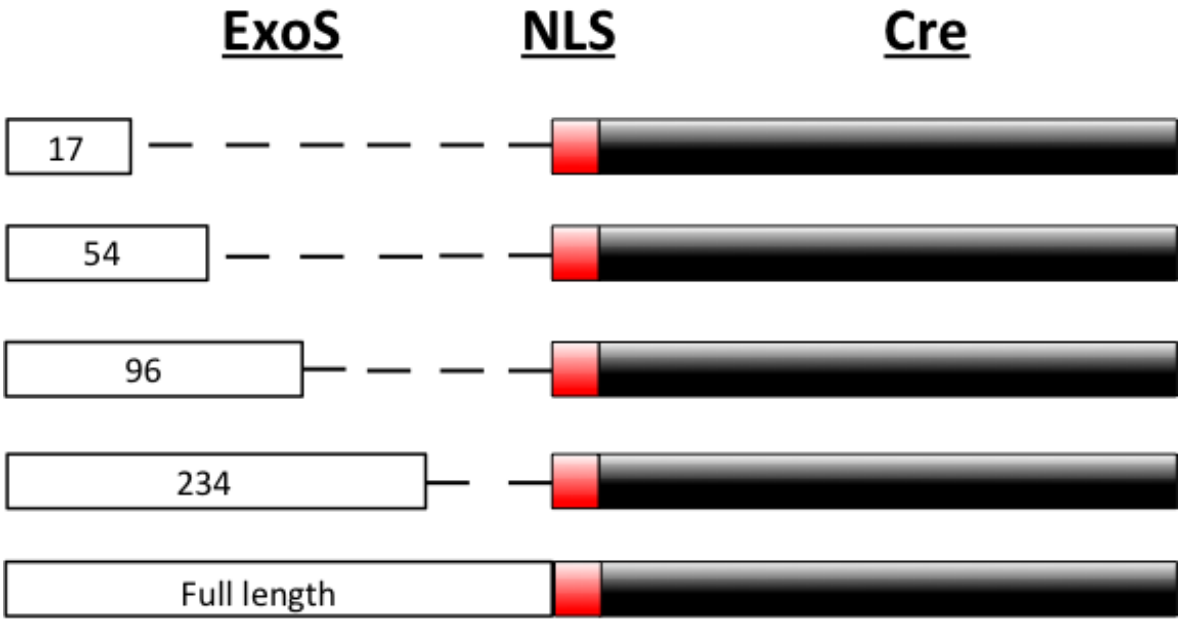

Figure 3-4. Constructs containing variable regions of ExoS fused in-frame with Cre recombinase. Various lengths of ExoS were PCR amplified and fused in-frame with a nuclear localization sequence followed by Cre-recombinse.

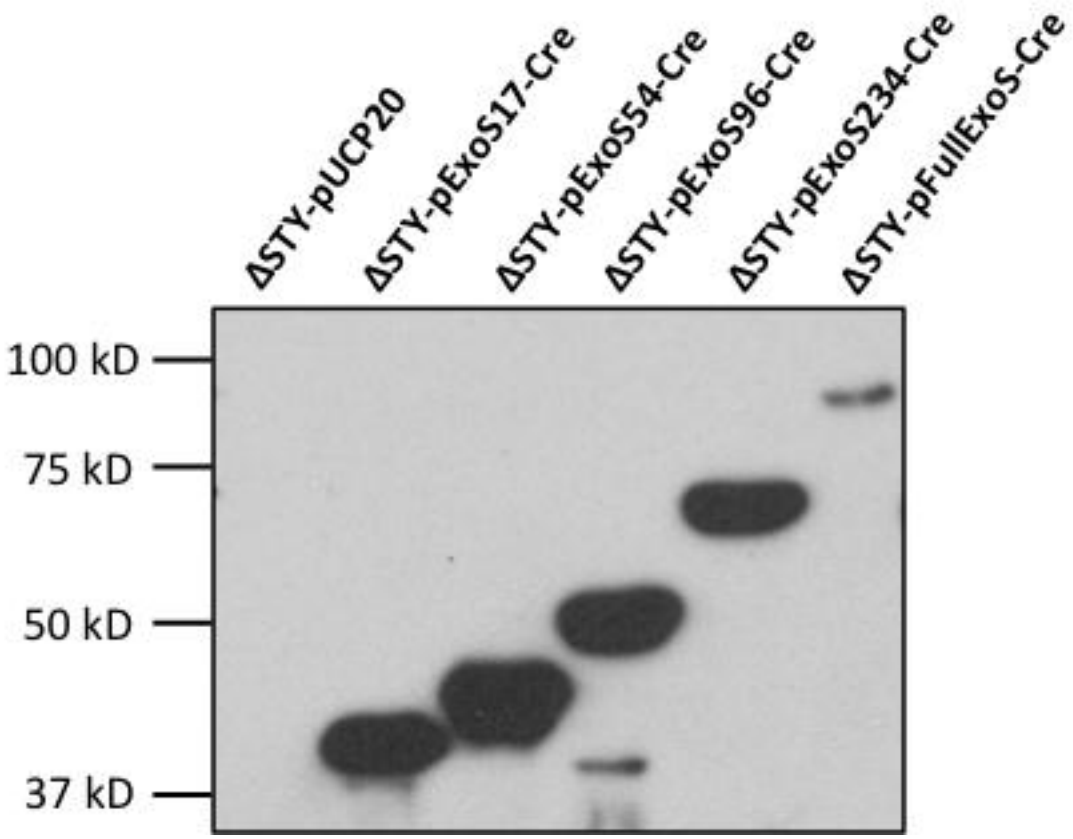

Figure 3-5. Production of ExoS-Cre fusion proteins by *P. aeruginosa* strain PAK-JΔ*STY*. Bacteria were grown under type III inducing conditions for 3 hours at 37°C. Bacteria were collected, pelleted, and the supernatants were removed. Pellets were lysed by adding protein sample buffer and subject to Western blotting with an antibody against Cre-recombinase.

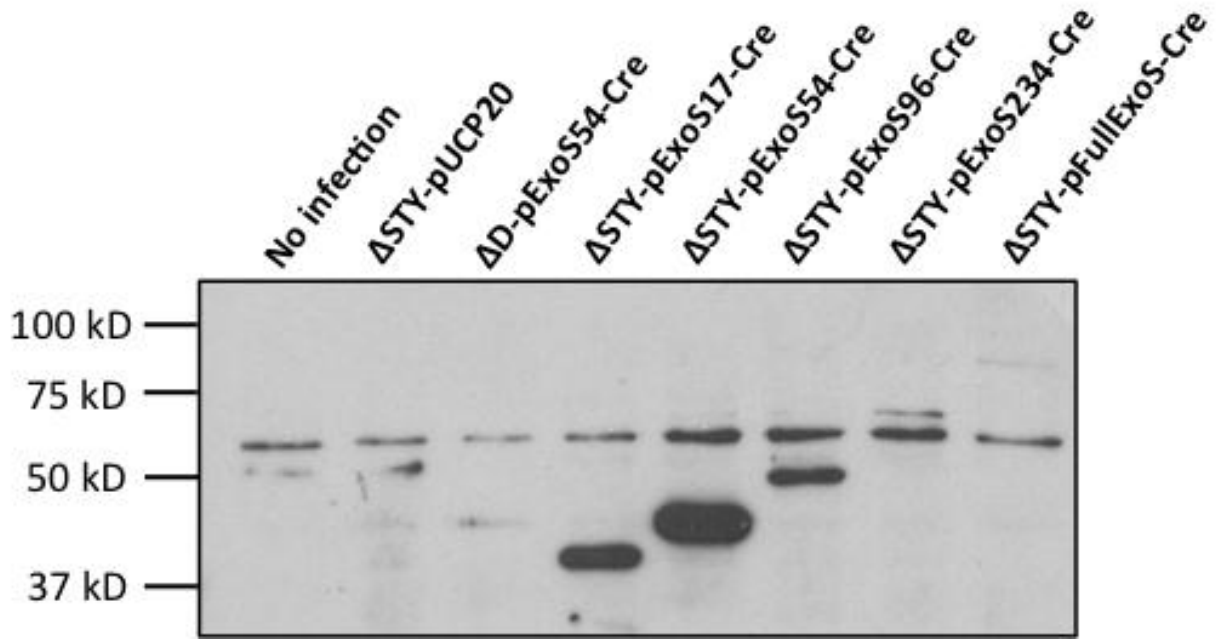

Figure 3-6. Injection of ExoS-Cre fusion proteins via the bacterial T3SS. Te26 cells were infected with strains expressing the various ExoS-Cre fusion proteins for 3 hours at an MOI of 100. Following infection, bacteria were cleared and the cells were collected and lysed. Cell lysates were then subjected to Western blotting with an antibody recognizing Cre-recombinase.

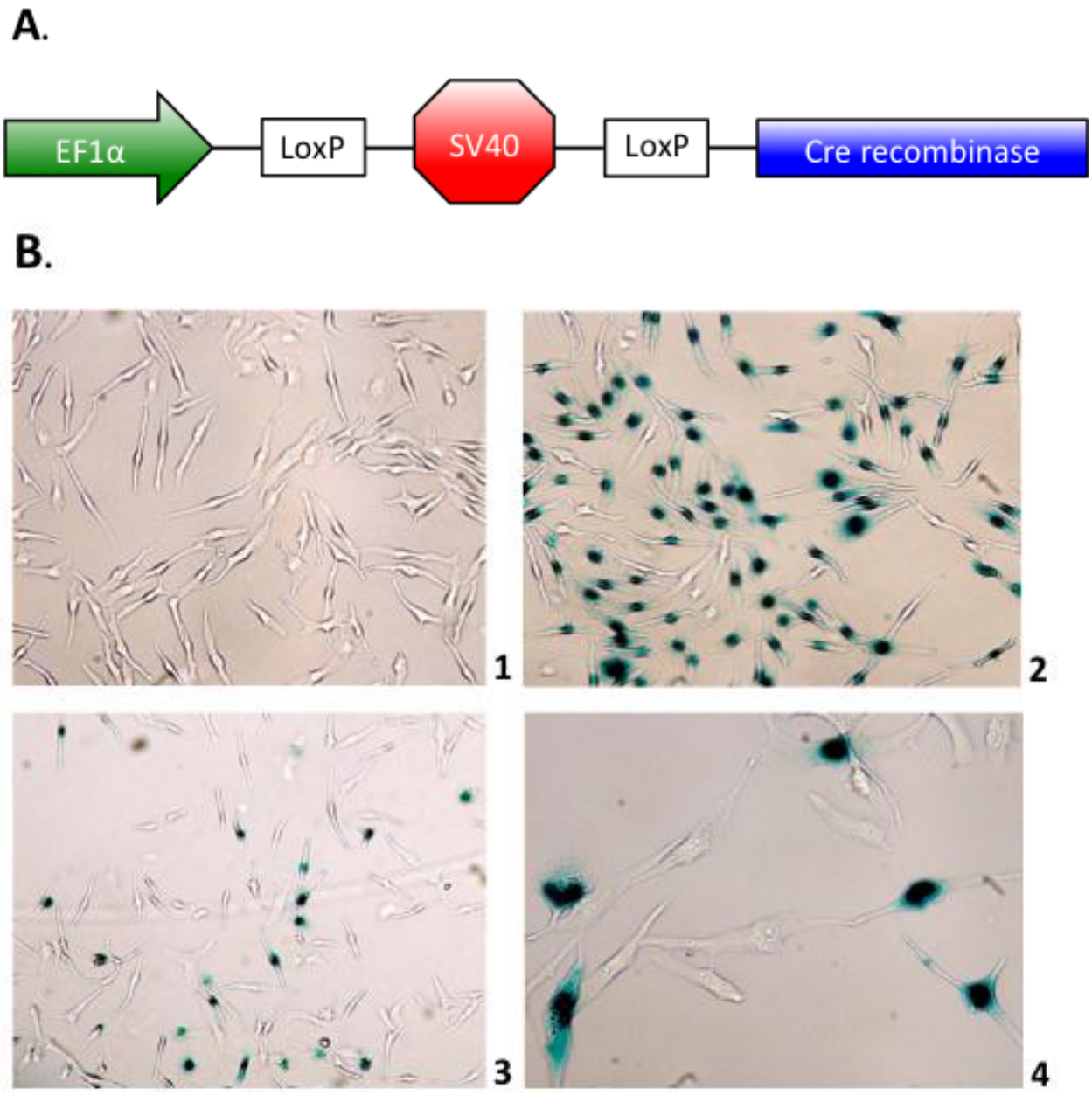

Figure 3-7. Bacterial delivered Cre-recombinase is functional. (A) Schematic illustrating the transcpritional terminator blocking *lacZ* expression in Te26 cells. (B) Infection of Te26 cells with PAK-JΔ*STY* expressing ExoS54-cre. 1.) Non infected T226 cells. 2.) Te26 cells with lenti-virus expressing cre recombinase. 3.) Infection with PAK-JΔ*STY* expressing ExoS54-cre for 3 hours at an MOI of 50. 4.) Close up view of bacterial infected cell showing nuclear localization of β-galactosidase.

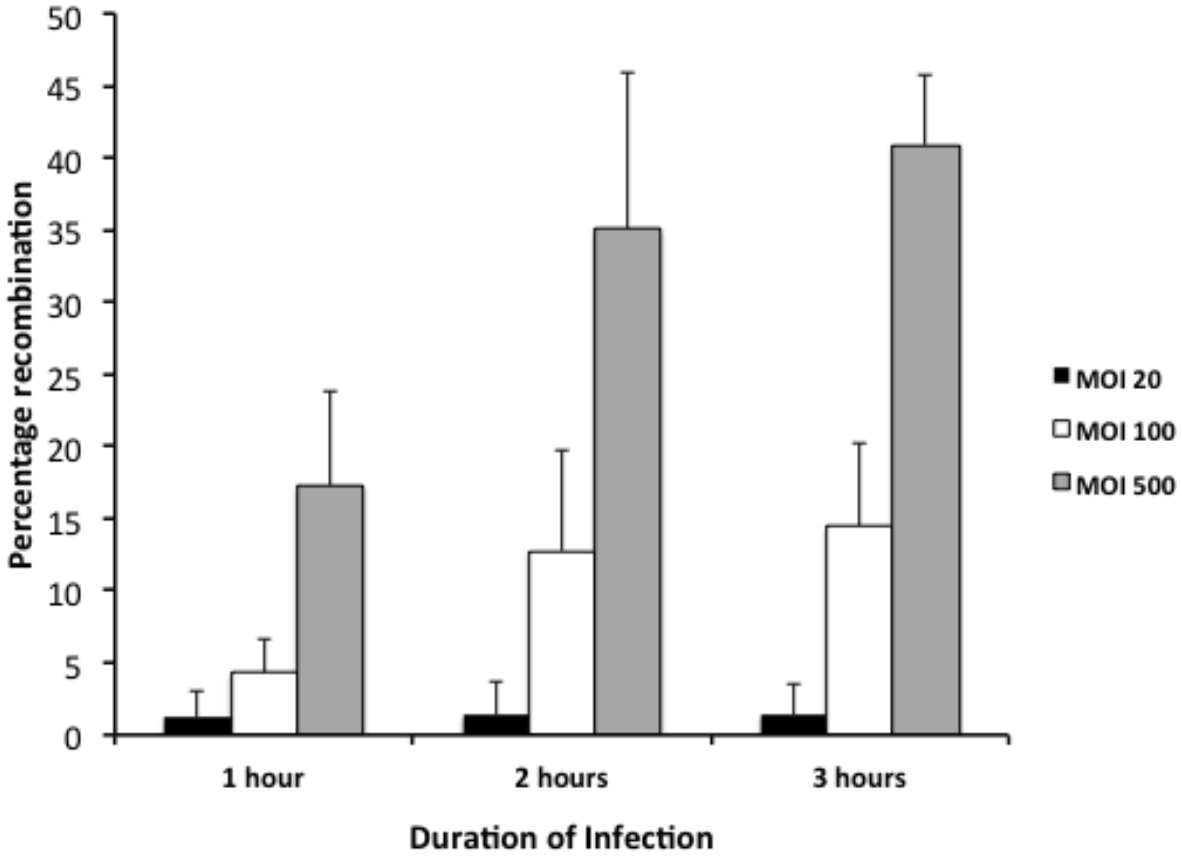

Figure 3-8. β-galactosidase expression resulting from bacterial delivered Cre-recombinase is dose dependent. Te26 cells were incubated with PAK-JΔ*STY* expressing ExoS54-Cre for various times and MOIs. Following infection, bacteria were stained for β-galactosidase and the number of positive cells was counted.

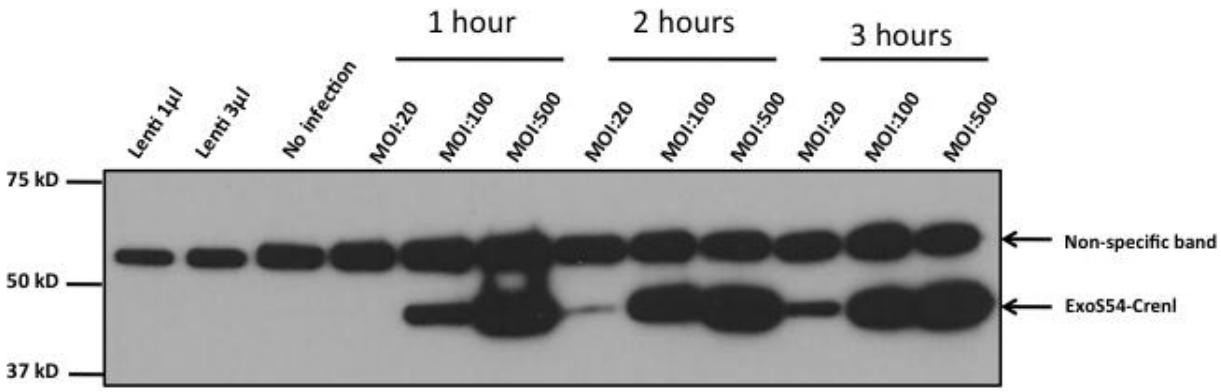

Figure 3-9. Injection of ExoS54-Cre is dose dependent. Te26 cells were infected with PAK-JΔ*STY* containing ExoS54-Cre for various times and MOIs. Cells were collected, lysed and subject to western blotting with an anti-Cre antibody. Cells were also infected with 2 different doses of lenti-virus expressing Cre-recombinase for 48 hours. The cells were then collected, lysed and subject to western blotting.

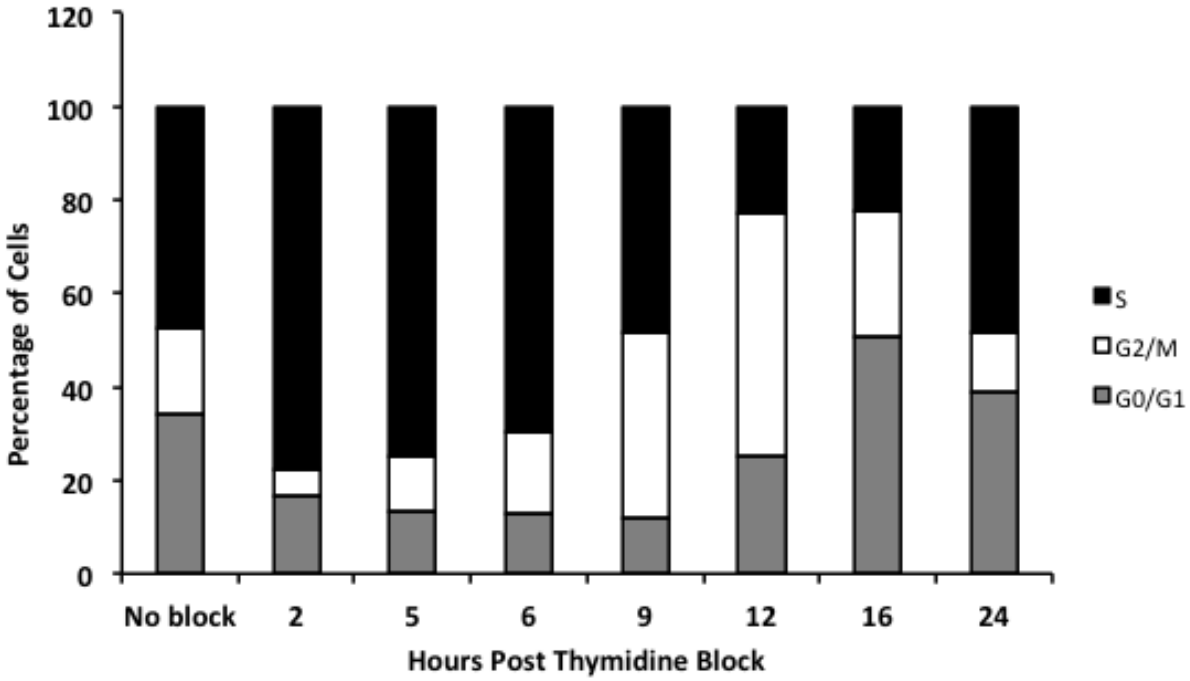

Figure 3-10. Synchronization of Te26 cells using a double thymidine block. Te26 cells were synchronized using a double thymidine block and then released. Cells were collected at various time points post blocking and subjected to flow cytometry to determine the number of cells in each phase of the cell cycle.

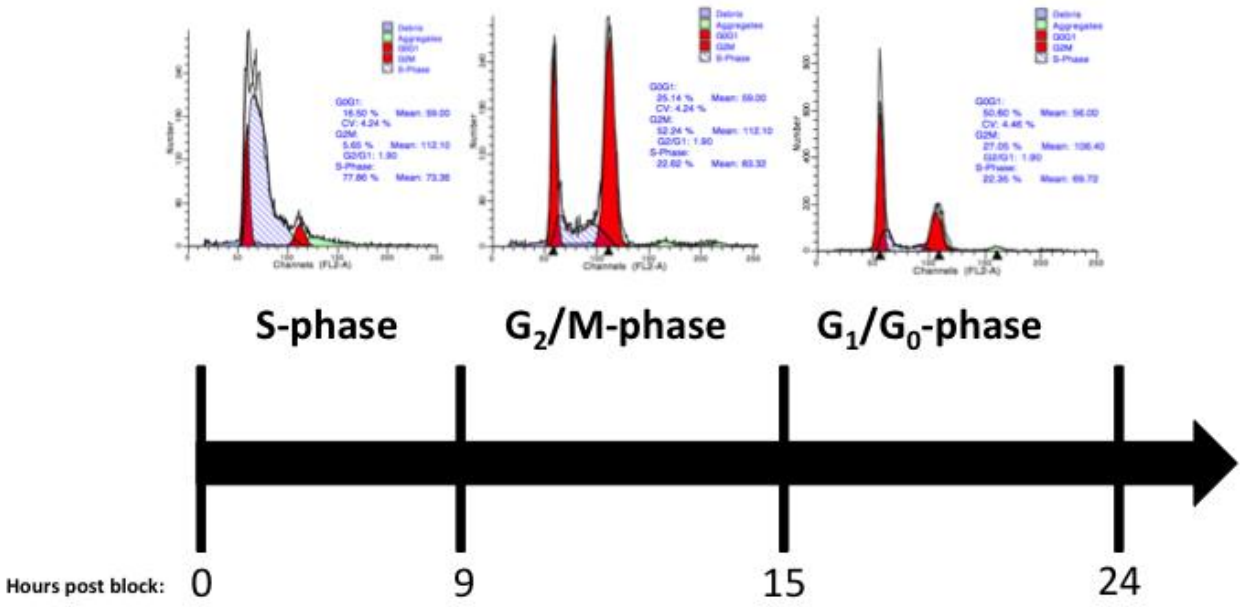

Figure 3-11. Time-line displaying the length of each phase of the Te26 cell cycle. Based on the results from the synchronizing of Te26 cells, a timeline was created to indicate the length of each phase of the cell cycle. This was the basis for infections during the different phases of the cell cycle.

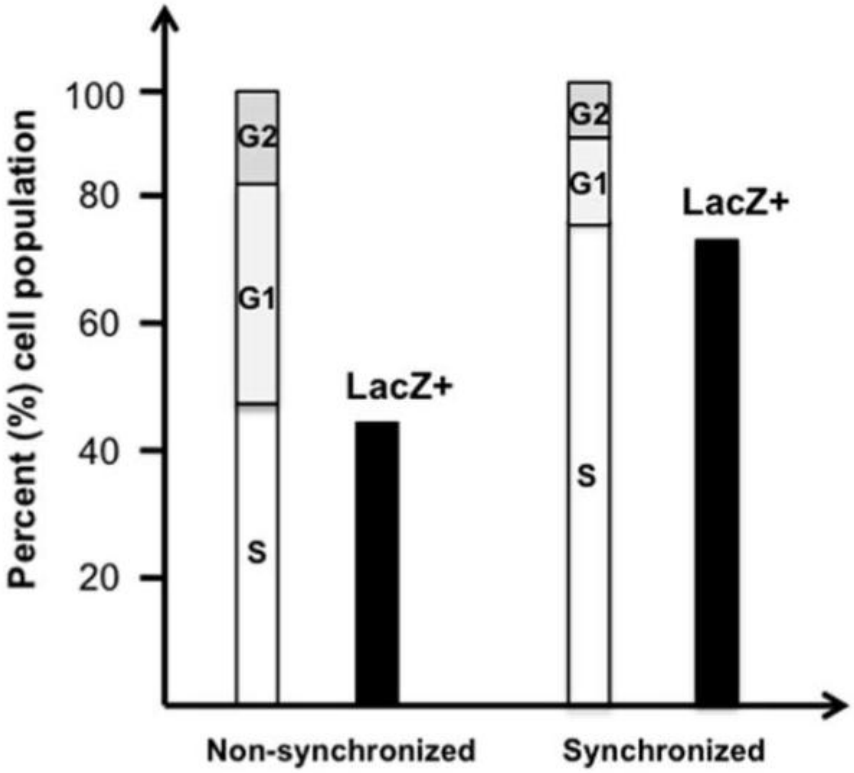

Figure 3-12. Cell cycle influences the recombination efficiency of bacterial delivered Cre-recombinase. Non-synchronized cells were infected with PAK-JΔ*STY* expressing ExoS54-Cre for 3 hours at an MOI of 500 and cells were then stained for β-galactosidase. Te26 cell synchronized to the S-phase of the cell cycle were infected under the same conditions and also stained for β-galactosidase. Graph compares the number of β-galactosidase positive cells to the number of cells in S-phase.

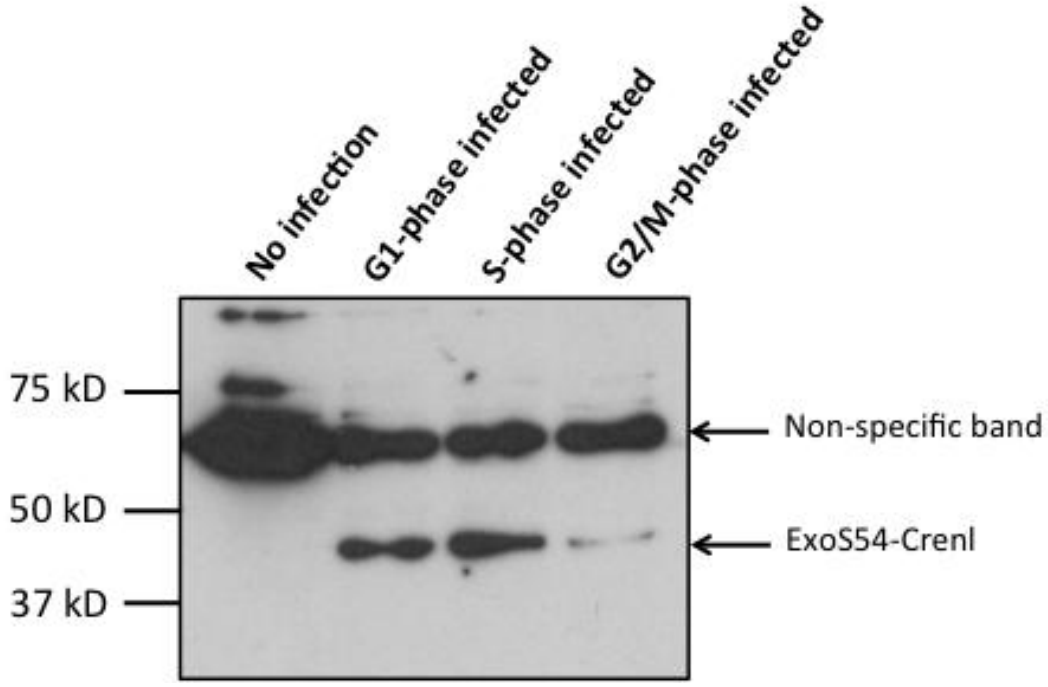

Figure 3-13. Injection of ExoS54-Cre during various phases of the cell cycle. Te26 cells were subject to a double thymidine block for synchronization. Afterwards, cells were infected at an MOI of 100 for 3 hours during the appropriate phase. Cells were collected, lysed, and subject to Western blotting with an antibody against Cre-recombinase.

CHAPTER 4
A NOVEL CYTOTOXIN REQUIRING BOTH TYPE I AND TYPE III SECRETION SYSTEMS FOR INTRACELLULAR DELIVERY

Background

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen responsible for causing diseases in immunocompromised individuals, most notably those suffering from severe burns or cystic fibrosis (100). In order to maintain infection, *P. aeruginosa* relies on the production of numerous virulence factors, some of which are injected directly into host cells via the cell contact mediated T3SS (33, 49). The T3SS is a proteinaceous needle, which translocates proteins, known as effectors, directly from the bacterial cytoplasm into the host cell (65). The effectors secreted by the T3SS of *P. aeruginosa*, Exoenzymes S, T, Y, and U, are the major contributors to cytotoxicity during the course of an infection (38, 151).

P. aeruginosa is able to inject copious amounts of protein in a relatively short period of time in order to fend off the host immune system. Our laboratory has recently demonstrated that T3SS of *P. aeruginosa* could be harnessed to deliver functional nuclear proteins into pluripotent and differentiated cells by using a laboratory strain derived from PAK, known as PAK-J, which displays a much higher secretion of effectors than any previously characterized strains of *P. aeruginosa* (16). The strain used to deliver nuclear proteins, PAK-JΔ*STY*, was devoid of all three known type III secreted exotoxins so as to reduce cytotoxicity to the host cells. Despite the depletion of the type III toxins, cytotoxic effects were still observed in cells after prolonged exposure to the bacteria. In an effort to minimize toxicity from additional virulence factors, a strain was created from PAK-JΔ*STY*, known as PAK-JΔ7, which is further defective in both type II secretion and quorum sensing. Although this strain had the major virulence factors

removed, it too showed toxicity when incubated with cells. In contrast, mutants that are defective in the T3SS display greatly reduced cytotoxicity, suggesting the presence of additional effectors secreted by the T3SS, however these proteins only play a major role in virulence when the known effectors are absent.

In an effort to identify additional type III secreted effectors, we found that nucleoside diphosphate kinase (NDK) is injected into HeLa cells by strains lacking ExoS, T, and Y. NDK is an ATP-utilizing enzyme that is secreted by the T1SS in *P. aeruginosa* and has been shown to cause cytotoxicity when incubated with macrophages (80, 181). The exact mechanism of toxicity is not currently understood, however, the working hypothesis is that NDK disrupts extracellular ATP concentrations that macrophages need for survival during the course of infection. In this report, we demonstrate for the first time that NDK is not only able to cause cytotoxicity when expressed in HeLa cells, but can also be translocated into host cells via the T3SS. Evidence is presented to support T1SS-dependent NDK secretion followed by T3SS-dependent injection into the host cells, suggesting a novel route of injection for the NDK protein during host-pseudomonas interaction.

Materials and Methods

Generation of Flag-Tagged NDK Constructs

The primers used to generate Flag-tagged versions of NDK are listed in Table 2-3. Human and *E.coli ndk* genes were PCR amplified and cloned into the pUCP20 vector using *Bam*HI and *Hind*III restriction sites located at the end of each primer. *ndk* from *P. aeruginosa* was cloned in pUCP20 using *Eco*RI and *Pst*I restriction sites and was further cloned into the eukaryotic expression vector pCDNA3.1(+) using *Bam*HI and

*Eco*RI sites. All constructs were verified using restriction enzyme digestions as well as DNA sequencing.

Protein Secretion Assay

Bacterial strains were grown overnight in 1.0 ml of L broth containing carbenicillin at 37°C. Overnight cultures were then inoculated at 5% into fresh L broth containing antibiotics for non-type III inducing conditions and L broth plus antibiotics and 5mM EGTA for type III inducing conditions. *P. aeruginosa* strains were grown in a shaking incubator at 37°C for three hours, after which bacteria were collected and spun down at 20,000xg. Bacterial supernatants were collected, mixed with equal volumes of protein sample buffer, and boiled for 10 minutes before subjecting to SDS PAGE analysis.

Protein Injection Assay

HeLa cells were seeded onto 6 well plates at approximately 70% confluency (8.4 x 10^5 cells) in medium containing antibiotic the night before infection. Two hours prior to infection, cells were washed twice in PBS and replaced with medium lacking antibiotics. Bacterial strains were grown in L broth supplemented with carbenicillin at 37°C until the OD_{600} reached 0.8. For an MOI of 20, 2 x 10^7 CFU per ml were incubated with HeLa cells for 4 hours. Following infection, bacteria were washed and cells were harvested by trypsinization. Cells were spun down at 500xg for 5 minutes and then washed with PBS 3 times. After centrifugation, HeLa cell pellets were suspended in 40µl of 0.25% Triton-X 100 and placed on ice for 10 minutes. Cell lysates were then centrifuged at 20,000xg for 2 minutes and the supernatants were mixed with equal amounts of 2x protein sample buffer. Samples were boiled for 10 minutes and then saved at -20°C until use.

Cytotoxicity Assay

Cells were infected with *P. aeruginosa* at indicated MOIs for indicated period of time as described above. Following infection, cells were washed three times with PBS to remove non-adhered cells and then adhered cells were collected by incubating with 0.25% trypsin for 5 minutes. Cells were then mixed and viable cells were counted using a hemocytometer.

Immunofluorescent Staining

Overnight bacterial cultures were inoculated into fresh L broth at a 1:50 dilution and allowed to grow for 2 hours at 37° C. 200µl of each bacterial strain was collected and pelleted by centrifugation at 20,000xg for 2 minutes. Pellets were washed once in PBS and suspended in a final volume of 100µl of PBS. To permeabilze bacteria, a 1% Triton-X 100 was added for 5 minutes. Samples were then transferred to glass coverslips and blocked in a solution containing 3% BSA and 10% NGS in PBS for 1 hour at room temperature. Samples were then incubated with primary antibody (α-FLAG 1:400) for one hour followed by 3 washes with PBS. Bacteria were then incubated with secondary antibody for 1 hour, washed in PBS, and stained with DAPI. Stained cells were then visualized under a fluorescent microscope.

Results

Strains Lacking Known Type III Secreted Effectors Still Cause Cytotoxicity

Studies in our laboratory have demonstrated that *P. aeruginosa* is capable of delivering functional Cre recombinase to pluripotent and differentiated cells when it was fused to the first 54 amino acids of the type III effector ExoS (16). We generated a strain known as PAK-JΔ*STY*, which is devoid of the three known type III secreted effectors, to use as the strain for protein delivery. Although this strain shows reduced cytotoxicity

when incubated with cells for less than three hours, observations from longer infection times revealed this strain possesses residual toxicity. In an effort to determine the degree of cytotoxicity resulting from longer incubation times with PAK-JΔ*STY*, we compared the number of HeLa cells that were adhered to tissue culture plates following infection by the wild-type PAK-J or PAK-JΔ*STY*. Figure 4-1 shows that at 4 hours post infection, only 50% of the cells were still adhered to the plate following incubation with PAK-JΔ*STY* whereas incubation with the wild-type strain resulted in only 5% of cells still adhered. These results are in agreement with previous cytotoxicity studies, which showed *P. aeruginosa* strains lacking type III effectors caused high levels of LDH release from cells following infection (95, 162). The T3SS is known to be a major virulence factor in *P. aeruginosa* infections, so a type III defective mutant was also incubated with HeLa cells. Cytotoxicity is dramatically reduced in HeLa cells incubated with PAK*ΔpscF,* as there is only a 10% reduction in the amount of adhered cells compared with a non-infected control (Figure 4-1). These results suggest that the T3SS of *P. aeruginosa*, even in the absence of known effectors, is still capable of causing toxicity.

Although only four type III exotoxins have been identified in *P. aeruginosa* thus far, it is possible that additional proteins are secreted through the type III needle, especially in the absence of the major effectors. To investigate this possibility, we screened the supernatants from wild-type PAK-J and a mutant strain PAK-JΔ*STYN*, which lacks the type III secreted effectors as well as the cap protein of the needle, PopN. Bacterial strains were grown under type III inducing condition and the supernatants were TCA precipitated, run on an SDS-PAGE, and stained with Coomassie blue. The goal of this

experiment was to identify novel protein bands that were present only in the strain lacking the type III secreted effectors. As seen in Figure 4-2, wild-type PAK-J, contains an upper segment of bands, corresponding to ExoS, T, and Y. PAK-JΔ*STYN* however, has an additional band present, indicated by the bottom arrow in Figure 4-2, suggesting the protein is secreted in higher amounts. The PopN protein is believed to be the cap of the type III injectisome and its deletion results in a strain that constitutively secretes effector proteins (178). MassSpec analysis of the band revealed the protein to be nucleoside diphosphate kinase (NDK), a protein previously characterized to be secreted through the T1SS of *P. aeruginosa* (80).

NDK is Secreted by Non-Mucoid Strains of *P. aeruginosa*

Previous reports have indicated that mucoid producing strains of *P. aeruginosa* are able to secrete NDK through a T1SS-dependent manner, whereas non-mucoid strains such as the common laboratory strain PAO1 are not (181). Based on the results from Figure 4-1, we set out to determine if NDK was secreted by the non-mucoid strain PAK-J, and whether the T3SS plays a role in the secretion process. To accomplish this, a plasmid containing a C-terminal FLAG-tagged form of the NDK from *P. aeruginosa* (pPaNDK) was introduced into various exotoxin deletion derivatives of PAK-J. The purpose of testing these mutations was to assess if the known type III effectors have any role in the secretion of NDK. Protein secretion was determined by growing bacterial cells in L-broth or L-broth containing 5mM EGTA for 3 hours at 37°C. Bacterial supernatants were collected, run on an SDS-PAGE gel, and subjected to Western Blotting with an antibody against the FLAG tag. Figure 4-3 shows that all of the strains secreted similar amounts of NDK under type III inducing and non-inducing conditions, suggesting the T3SS does not play a role in secretion of the protein. Additionally,

deletion of exotoxins does not appear to have any affect on the secretion of NDK as all of the strains secrete similar levels. Further evidence suggesting that the T3SS does not have a role in the secretion of NDK is demonstrated by the observation that the type III defective mutant, PAKΔ*pscF*, secretes NDK at amounts similar to strains possessing a functional T3SS. These results do, however, indicate that the non-mucoid strain PAK-J is able to secrete NDK, presumably via its T1SS.

NDK is Injected into Eukaryotic Cells in a T3SS Dependent Manner

Although the secretion data in Figure 4-3 suggest that the T3SS does not play a role in the secretion of NDK, it was possible that the amount of NDK secreted by the T3SS was so low that no detectable difference could be observed between type I and III secreted protein using Western blotting. If NDK is secreted through the injectisome, then it should behave like other type III effectors and be injected into eukaryotic cells. We therefore used the same deletion strains harboring FLAG-tagged NDK and incubated them with HeLa cells for three hours at MOI 20. Following infection, bacteria were cleared and the cells were lysed, followed by Western Blotting with an anti-FLAG antibody. Figure 4-4A shows that PAK-JΔ*STY* injects the greatest amount of NDK into HeLa cells and the amount injected is reduced as more exotoxins are present, with none being injected from the wild type PAK-J strain. These results suggest that amount of NDK injected is negatively influenced by the presence of other known type III effectors. A functional T3SS is required for translocation as infection with PAK-JΔ*pscF* shows trace amounts of NDK injection. It is possible that the faint band represents NDK that was taken up by pinocytosis or by an additional unknown mechanism. To confirm these results were not cell type dependent, we carried out the same infection in H1299 cells and observed the same results (Figure 4-4B). Taken together, the results in Figure

4-4 show that NDK is secreted by a T1SS, yet injection into HeLa cells is dependent on a functional T3SS. Also, the amount of NDK translocated into HeLa cells is elevated in bacteria lacking the type III effectors, suggesting these may play an inhibitory role, likely through competition, in the secretion of NDK under normal infection conditions.

NDK is Cytotoxic to Eukaryotic Cells

NDK has been reported to cause cytotoxicity in macrophages by disrupting ATP concentrations outside of the cell (181) however, it has not been determined whether bacterial NDK is capable of eliciting a cytotoxic response when expressed inside host cells. To examine this, NDK was cloned into a eukaryotic expression vector and co-transfected into HeLa cells with a plasmid expressing GFP (pEGFP). If NDK generated a cytotoxic response, the number of GFP positive cells should be lower in samples that were co-transfected with NDK when compared to cells transfected with GFP and pCDNA3.1 vector control. Forty-eight hours post-transfection, HeLa cells were collected and subjected to flow cytometry to quantify the amount of GFP positive cells. Figure 4-5 shows transfection with GFP and empty vector together resulted in 38% of HeLa cells expressing GFP, whereas co-transfection with NDK and GFP resulted in a 12% reduction in the amount of GFP positive cells. GFP was also co-transfected with a plasmid expressing the acute type III secreted cytotoxin ExoS, resulting in an 80% decrease in GFP positive cells compared to GFP and vector only transfected cells (Figure 4-5). Although NDK caused a significant reduction in the amount of GFP positive cells, the toxicity was not as robust as ExoS. This would suggest that NDK is not an acute toxin, but in the absence of other type III effectors it does play significant role in toxicity.

To further assess the cytotoxicity resulting from NDK, we deleted the gene via homologous recombination in a laboratory strain, PAKΔ7, resulting in the strain PAKΔ8. Strain PAKΔ7 has the type III exotoxins deleted, as well as the type II secretion system and quorum sensing (Table 2-1) in an effort to reduce cytotoxicity in eukaryotic cells. To determine if NDK expressing strains possess greater toxicity, HeLa cells were infected with PAKΔ8/pUCP20, PAKΔ7/pUCP20, and PAKΔ8/pPaNDK for five hours. Bacteria were then cleared by washing with PBS and fresh media containing antibiotics was applied to the cells to eliminate any residual bacteria. Twenty-four hours later, lifted cells were washed away and the remaining adhered cells were trypsinized and counted using a hemocytometer. Results from Figure 4-7 show that incubation with PAKΔ7 at an MOI of 100 resulted in a 75% reduction in the number of adhered cells compared to uninfected control. However, incubation with PAKΔ8 resulted in only a 25% decrease in adhered cells. Complementation of PAKΔ8 with a wild type *ndk* gene restored toxicity, causing a similar amount of cell lifting as infection with the PAKΔ7. NDK containing strains do elicit a toxic response however, the toxicity is not as robust and the time necessary to cause toxicity is much longer compared to the type III secreted effector ExoS. These results not only indicate that strains possessing NDK result in higher toxicity toward eukaryotic cells, but that this response is delayed in comparison to other known type III secreted effectors.

Previous reports have demonstrated that NDK from *Mycobacterium tuberculosis* is also responsible for causing toxicity when incubated with eukaryotic cells (23, 24). We were curious as to whether this was a general feature of NDKs or relevant only to *P. aeruginosa* and *M. tuberculosis*. To examine this, NDKs from *E.coli* (pEcNDK) and

humans (NM-23H2) (pHuNDK) were cloned into pUCP20 and transformed into *P. aeruginosa*. NDKs from *E. coli* and *P. aeruginosa* share 60 percent amino acid homology while the human form is 42 percent homologous to *P. aeruginosa* (22). As shown in Figure 4-6A, NDKs from *E. coli* and humans are also secreted from *P. aeruginosa* in a type III-independent manner. Testing these strains for protein injection revealed that NDK from *E.coli* was injected at amounts similar to *P. aeruginosa* NDK, whereas the human form showed a much lower amount (Figure 4-6B). The toxicity assay results in Figure 4-7 reveal that strains possessing *E. coli* and human NDKs also cause HeLa cells to detach from the monolayer, with 75% detaching from *E. coli* NDK and roughly 65% from the human form. Interestingly, the human form of NDK is secreted at levels similar to the other proteins however the amount injected is greatly reduced, possibly due to the lack of necessary signal for injection from the type III needle. Although the human NDK causes a cytotoxic response, it is slightly reduced compared to that from *E. coli* and *P. aeruginosa* which probably arises from the observation that human NDK is not as readily injected. Based on these observations it is feasible that a relatively low amount of injected human NDK is necessary to elicit a cytotoxic response. When taken together, these results suggest that NDKs from *E.coli* and humans are able to generate a cytotoxic response when injected into eukaryotic cells.

Kinase Activity of NDK is not Required for its Cytotoxicity

Nucleoside diphosphate kinase is responsible for generating nucleotide triphosphates (NTPs) from nuclotide diphosphates (NDPs) by transfer of a terminal phosphate from an NTP to an NDP (147). To date, all known prokaryotic and eukaryotic NDKs possess a conserved histidine residue (H117) that becomes phosphorylated

during the generation of NTPs (22). We therefore wanted to determine whether the phosphorylation was responsible for the toxicity elicited by NDK. To accomplish this, histidine H117 of the *P. aeruginosa* NDK was mutated to glutamine (H117Q) through site directed mutagenesis. As seen in Figure 4-5, co-transfection of the mutant NDK (pNDKH117Q) with GFP resulted in an 18 percent reduction in the amount of GFP positive cells when compared to co-transfection with the vector control. Surprisingly, the toxicity was greater in the mutant than in the wild-type, suggesting that toxicity from NDK is not linked to the kinase activity. Infection with the *P. aeruginosa* strain harboring the mutated NDK resulted in a toxic effect similar to that of other NDK containing strains (Figure 4-7). Taken together, these assay results suggest that the toxicity from NDK on mammalian cells is independent of its kinase domain.

Secretion of NDK by the T1SS is Necessary for Injection

To investigate if secretion of NDK by the T1SS was necessary for injection into HeLa cells by the T3SS, we generated a truncated version of NDK that is unable to be secreted through the T1SS (Figure 4-8A). The truncated form lacks the last eight C-terminal amino acids, which has previously been shown to be essential for T1SS-dependent NDK secretion (80). The construct was then introduced into the PAKΔ8 background and tested for secretion under type III inducing condition as well as injection into HeLa cells. Figure 4-8B shows that the full length NDK was secreted into the extracellular media whereas the truncated form showed only a faint band. This secretion was independent of type III inducing conditions, and was shown not to be a result of higher protein expression from the strain containing the full-length protein, as the truncated form was produced at similar amounts (Figure 4-8B). Although the truncated from of NDK appears to run at a higher molecular weight than the full-length form,

sequencing results confirm the protein sequence is correct. A possible explanation for this observation is that *P. aeruginosa* is somehow modifying the protein. These results are in agreement with a previous report, which demonstrated that NDK secretion through the T1SS is dependent on a DTEV amino acid motif located in the last 8 amino acids of the carboxyl terminus (80).

To determine if NDK secretion is necessary for injection via the T3SS, bacterial stains containing both forms of NDK were incubated with HeLa cells at an MOI of 20 for 3 hours. As shown in Figure 4-9, only the full length NDK was translocated into HeLa cells. The truncated protein showed faint or inconclusive bands suggesting these proteins are not readily injected into the host cells. Taken together, the above data suggest that type I secretion of NDK is necessary for injection by the T3SS.

Type I Secreted NDK is Injected via a Functional T3SS

Since the NDK type I secretion signal sequence is required for its type III mediated injection, it is possible that NDK is secreted into the extracellular space by the T1SS first, and then is somehow directed into the host cell by the T3SS. To test this possibility, a type III defective strain containing FLAG-tagged NDK (PAK-JΔ*pscF*/*pPaNDK*) was incubated with HeLa cells alone or in combination with varying MOIs of a strain containing a functional T3SS but lacking NDK-FLAG (PAK-JΔ*STY*) to investigate whether NDK-FLAG secreted through the T1SS of the Δ*pscF* strain could be injected via the functional T3SS of the PAK-JΔ*STY*. Figure 4-10A shows that HeLa cells incubated with both PAK-JΔ*pscF* and PAK-JΔ*STY* resulted in higher levels of NDK-FLAG injection in a PAK-JΔ*STY* dose dependent manner when compared to infection with PAK-JΔ*pscF* alone. Additionally, the same results were observed using another type III defective mutant, PAK-JΔ*exsA* (Figure 4-10B). ExsA is the master regulator of

the T3SS, and deletion results in a type III defective mutant (177). To demonstrate these observations were dependent on a functional T3SS, the same experiment was carried out using two type III defective strains. Figure 4-11 shows that the amount of NDK-FLAG present after infection with PAK-JΔ*pscF*/*PaNDK* does not increase with the addition of PAK-JΔ*exsA*, demonstrating that the previously observed increase in NDK injection was dependent on a functional T3SS. Together, the data presented in Figures 4-10 and 4-11 suggests that type I secreted NDK can be injected into HeLa cells in the presence of a functional T3SS.

NDK Localizes to the Outer Membrane of *P. aeruginosa*

The results from experiments thus far suggest that NDK is secreted by the T1SS, yet requires a functional T3SS in order to be translocated into eukaryotic cells. In order for this to occur, NDK must come in contact with the bacterial outer membrane or with one of the components of the T3SS. We therefore carried out an experiment to determine if NDK was localized to the outer surface of *P. aeruginosa.* A strain harboring flag tagged NDK (PAK-JΔ*ST*/pPaNDK) and another containing a flag tagged form of ExoS (PAK-JΔ*N*/pHW0224) were grown under type III inducing conditions for three hours. ExoS was chosen as a control protein due to the fact there has been no documented evidence suggesting that it localizes with the bacterial surface. Following growth, bacterial strains were immunostained without permeabiliziation on glass coverslips with an anti-Flag antibody and visualized under a fluorescent microscope. As seen in Figure 4-12A, the strain containing NDK stained positive, whereas the strain containing ExoS did not. DAPI staining of the bacteria show that the lack of signal in the ExoS possessing strain was not a result of a lack of bacteria. Permeabilzing the bacterial strains before staining however, results in positive Flag staining for both,

suggesting that NDK is in fact localized to the bacterial outer surface (Figure 4-12B). Higher magnification images of non-permeabilzed NDK containing strains reveal that the green signal is localized to the outer areas of the bacteria further suggesting that NDK is localized to the outer membrane (Figure 4-13).

Discussion

Previous studies, along with data presented here, clearly show that prolonged exposure to *P. aeruginosa* strains lacking type III effectors results in significant cytotoxicity (162). This toxicity is dependent on the T3SS, as infecting with strains lacking a functional T3SS are less cytotoxic. To date, only four exotoxins have been characterized in *P. aeruginosa*, which is a relatively low number compared to other T3SS containing bacteria, such as *Yersinia*, *Salmonella* and *Shigella* species (27). The goal of this study was to determine if any additional proteins are secreted through the T3SS in the absence of the known effectors. Examining secretions from a strain of *P. aeruginosa* lacking the type III secreted effectors and the wild-type revealed that NDK was secreted at higher levels when grown under type III inducing conditions.

Previous reports have shown that NDK is secreted via the T1SS of mucoid strains of *P. aeruginosa* however, no studies have demonstrated that NDK can be injected by the T3SS (80). While our results suggest that NDK is not secreted in a type III dependent fashion, they do demonstrate a functional T3SS is necessary for the injection of NDK into eukaryotic cells. Our findings are the first to report that NDK is injected into cells by the T3SS and that intracellular expression of NDK results in a cytotoxic response, although not as robust as that seen by the type III secreted effector ExoS. Additionally, toxicity does not appear dependent on NDK's kinase activity as transfection and infection data demonstrate the kinase defective mutant generates as

much toxicity as wild-type (Figures 4-5, 4-7). It is therefore possible that NDK from *P. aeruginosa* possesses additional functional domains that are responsible for the cytotoxicity. In the case of *M. tuberculosis*, in addition to its kinase activity, NDK has also been show to possess GAP activity for Rho-GTPases (23, 151).

NDK toxicity does not appear to be restricted to that of *P. aeruginosa*, as a NDK defective *P. aeruginosa* complemented by NDK from *E.coli* or humans also displayed similar toxicity (Figure 4-5). Although the human form of NDK is not as readily injected as NDKs from *P. aeruginosa* and *E.coli*, the toxicity was only slightly reduced (Figure 4-6) suggesting that only a small amount of NDK is necessary to generate a toxic response. Additionally, it is possible that expressing a functional NDK in *P. aeruginosa* results in the expression of additional unknown virulence mechanisms. Recent data from our laboratory show that *P. aeruginosa* strains expressing NDK are able to induce pro-inflammatory cytokine expression in human alveolar epithelium cells (Unhuan Ha, unpublished result). These results were seen in strains expressing the NDK from *P. aeruginosa, E. coli*, as well as human, but not in strains lacking NDK or lacking a functional T3SS, consistent with the current findings.

NDK does not possess a canonical T3SS signal sequence, however, by making a truncated form of NDK that is unable to be secreted by the T1SS, it was discovered that the same signal sequence is required for injection by the type III needle. There are two possible explanations for this observation (i) a type III chaperone protein recognizes this sequence and guides it to the injectisome or (ii) NDK must be secreted into the extracellular milieu first, and then is injected by the type III needle. The data presented in this study support the second explanation. Our secretion data show that NDK is

secreted in a type III independent fashion, suggesting that majority of the secreted protein results from the T1SS. Complementation studies revealed that NDK secreted from a T3SS defective mutant could be translocated into HeLa cells when incubated with a strain possessing a functional T3SS but lacking NDK (Figure 4-10). Additionally, extracellular complementation studies with two type III defective mutants failed to show an increase in NDK translocation (Figure 4-11), further suggesting the critical role of T3SS in NDK delivery.

The traditional model for type III injection suggests that effectors are injected directly from the bacterial cytoplasm into the host cell through the hollow needle (49). While the observations in this study are contrary to the classical model, they are not unprecedented. Vidal *et al* have published results on a similar phenomenon involving cooperation of the type V secretion system with the T3SS. In their report, it was shown that EspC of *E. coli* was secreted into the extracellular environment through a type V secretion system and then translocated into epithelial cells by the T3SS (167). However, the exact mechanism behind this observation is unknown, although they suggested EspC was able to bind to the type III needle before being translocated. A recent publication by Akopyan *et al* demonstrated that the type III effectors of *Yersinia pseudotuberculosis* were located on the outer membrane surface of the bacteria, as well as in the bacterial cytoplasm (3). They showed that effectors localized to the extracellular surface of the bacteria could be injected into eukaryotic cells in a type III dependent fashion. It was also shown that the signal required for injection from the bacterial cytoplasm was different from the sequence necessary for translocation from the bacterial surface. While their findings do not discredit the traditional method of

injection, they are evidence of an additional method by which effector molecules can be localized to the outer surface of the bacteria and be subsequently injected by the injectisome. Perhaps a specific signal sequence is needed for a protein to bind to the type III needle for injection to occur. This report shows that NDK from *E.coli*, which is 60 percent homologous to that of *P. aeruginosa*, is able to be secreted by the T1SS and injected into HeLa cells, whereas the human form can be secreted but not as readily injected. The human form is 42 percent homologous to *P. aeruginosa* and perhaps a lack of the necessary secretion sequence may account for its inability to be injected.

Results presented in this study suggest that NDK is secreted into the surrounding media and then localizes to the bacterial outer surface (Figure 4-12). This data supports the idea of surface localized effectors possessing the ability to be translocated into host cells via the T3SS. Based on this data, along with the observation from others, we propose a model suggesting how NDK or other surface localized effectors might be injected into host cells (Figure 4-14) (3, 167). During the course of infection, NDK is secreted by the T1SS and localizes to the bacterial outer membrane, where it can coat the bacterial surface (Figure 4-14 panels A, B). As the bacteria come in close contact with the target cell, type III needles begin to form underneath the effector protein bound to the bacterial surface and forces the protein into the host cell (Figure 4-14 panels C, D). This could be accomplished if NDK is able to bind to proteins located at the tip of the needle or can bind to the needle itself.

In summary, results from this study have identified NDK as an additional protein injected into eukaryotic cells by the T3SS. Although not as toxic as the known type III effectors, in the absence of said effectors, NDK is able to cause significant cytotoxicity.

We have demonstrated that NDK is secreted by a T1SS, yet is injected into HeLa cells in the presence of a functional T3SS. These results suggest NDK is not translocated by the traditional method of type III injection, but instead injected by a newly emerging model that happens in cooperation with the traditional one (124, 134). Further efforts are underway to determine the exact mechanism by which extracellular proteins can be injected by the T3SS and the mechanism by which the NDK causes toxic effect on host cells.

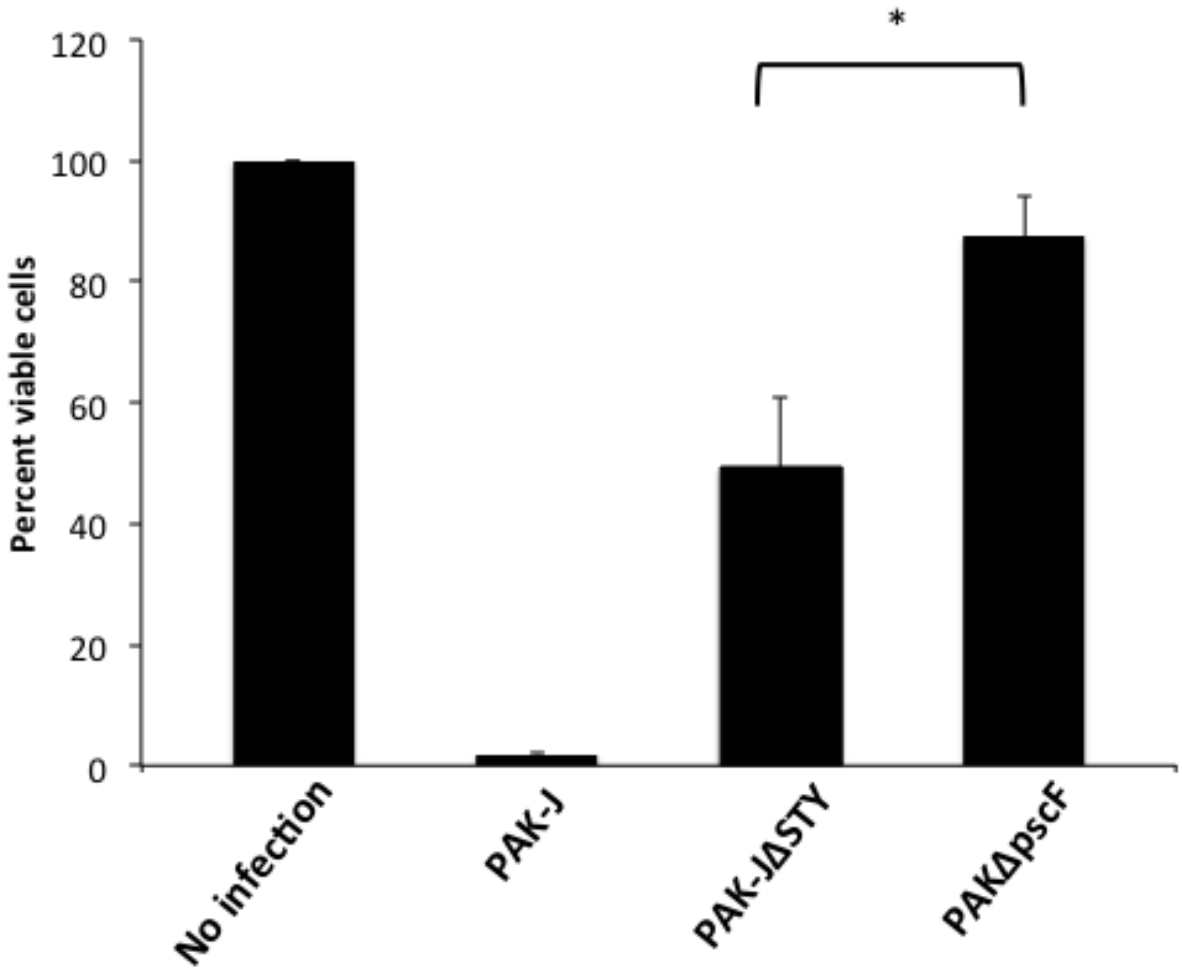

Figure 4-1. Strains lacking type III effectors are cytotoxic**.** HeLa cells were infected for 4 hours at an MOI of 50 with the indicated strains of *P.aeruginosa*. Following infection, floating cells were removed and remaining cells were collected and counted using a hemocytometer. Samples normalized to non-infected control. * p=0.007 using students T-test.

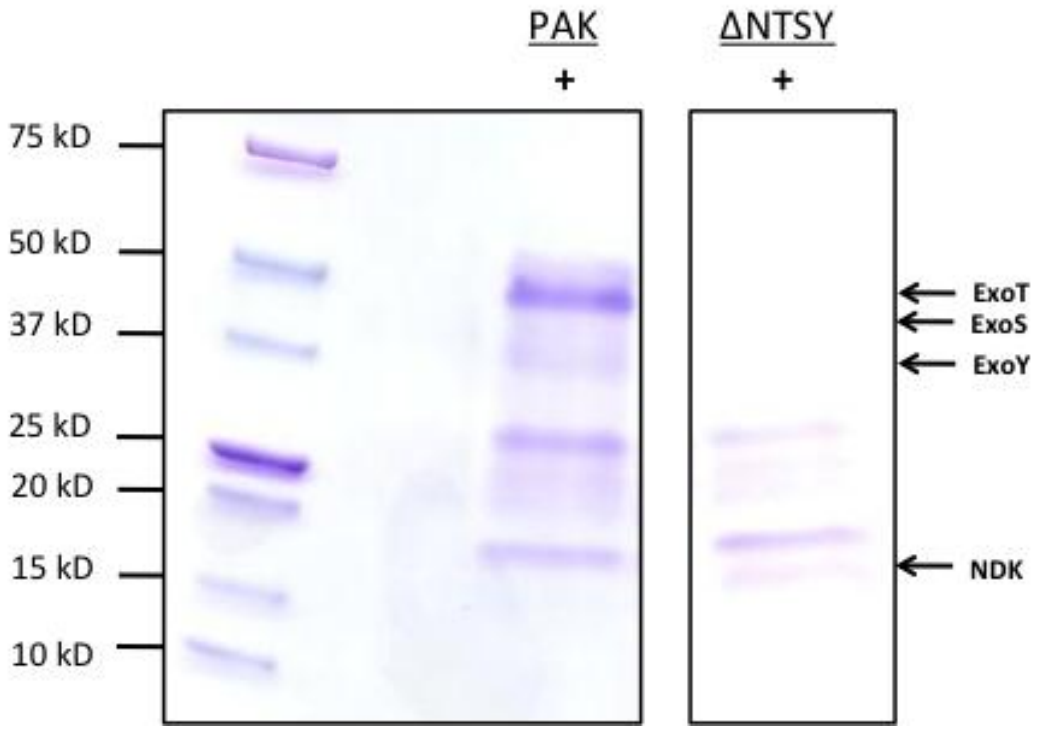

Figure 4-2. Secretion of NDK from *P.aeruginosa*. Bacteria were grown in 5mM EGTA for 3 hours. Bacteria were pelleted and supernatants were collected and subjected to TCA precipitation for 3 hours. Samples were then run on an SDS-PAGE and then stained with Coomassi blue.

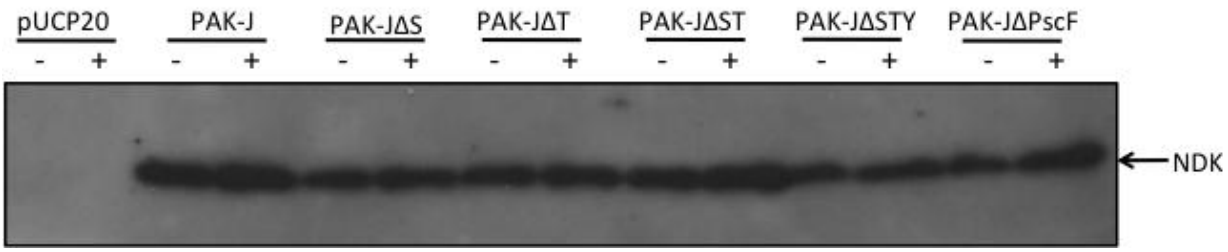

Figure 4-3. Non-mucoid strains of *P.aeruginosa* secrete NDK. Bacterial strains containing pPaNDK were grown under type III inducing and non-inducing conditions. Supernatants were collected and subjected to Western blotting with an antibody to the Flag tag.

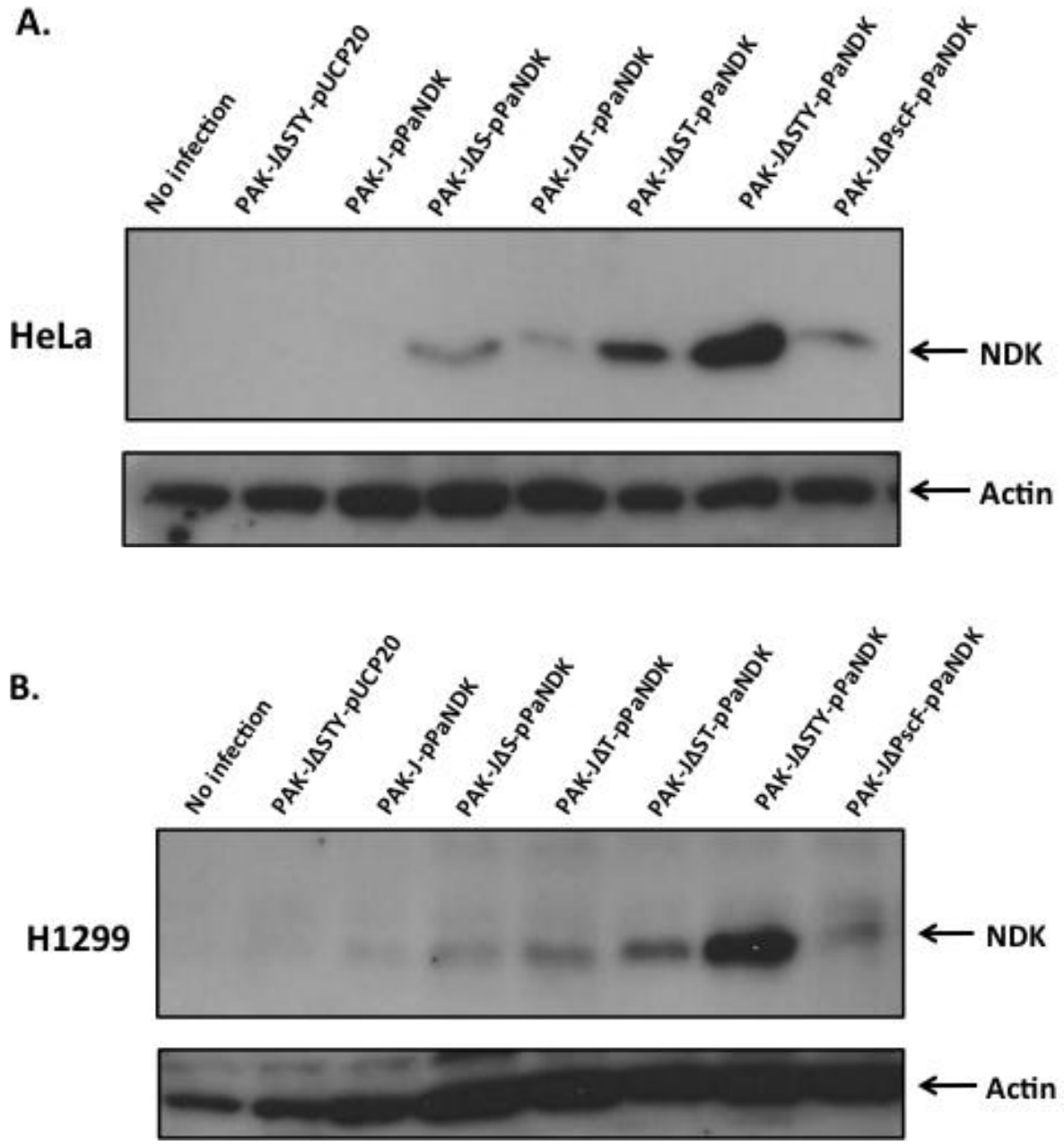

Figure 4-4. Injection of NDK into eukaryotic cells is dependent on the T3SS. (A) HeLa cells were infected with indicated bacterial strains for 3 hours at an MOI of 20. Cells were collected, lysed, and the resulting lysates were subjected to Western blotting with an anti-Flag antibody. (B) Westen blot of H1299 cells infected with the same strains above under the same conditions.

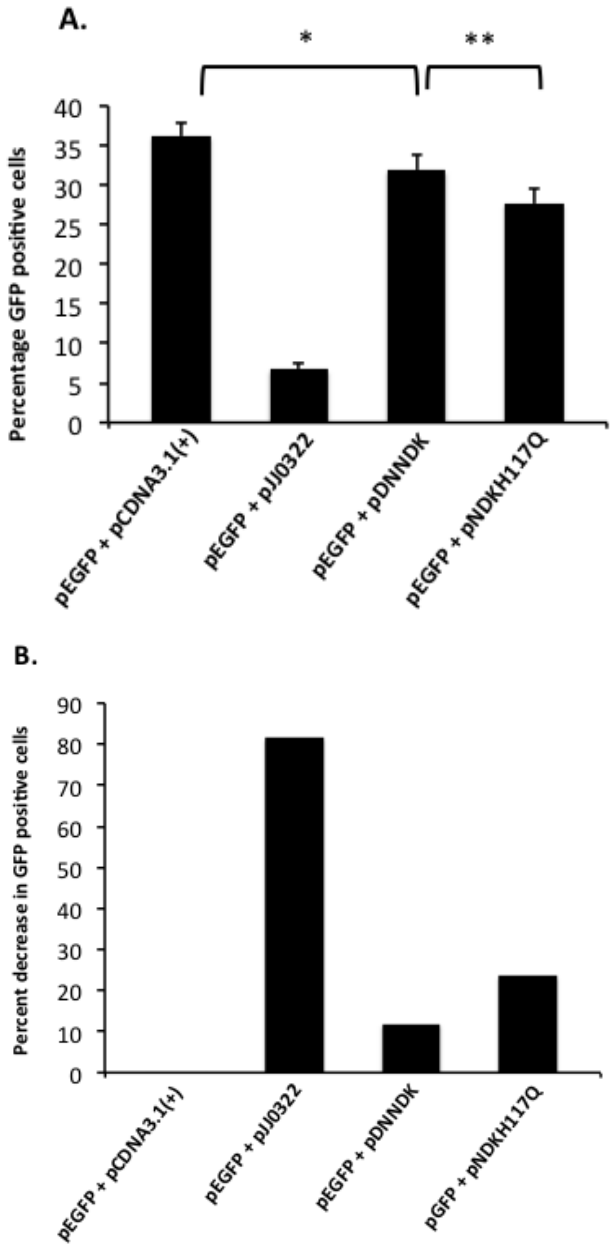

Figure 4-5. Cytotoxicity resulting from expression of intracellular NDK. (A) Cytotoxicity resulting from the expression NDK from *P. aeruginosa* from a eukaryotic expression vector. Plasmids containing GFP and NDK were cotransfected into HeLa and 48 hours later, the number of GFP positive cells was quantified using flow cytometery. * p=0.017 and **p=0.023 using students T-test. (B) Results from part A normalized to the vector control showing the percentage decrease of GFP positive cells.

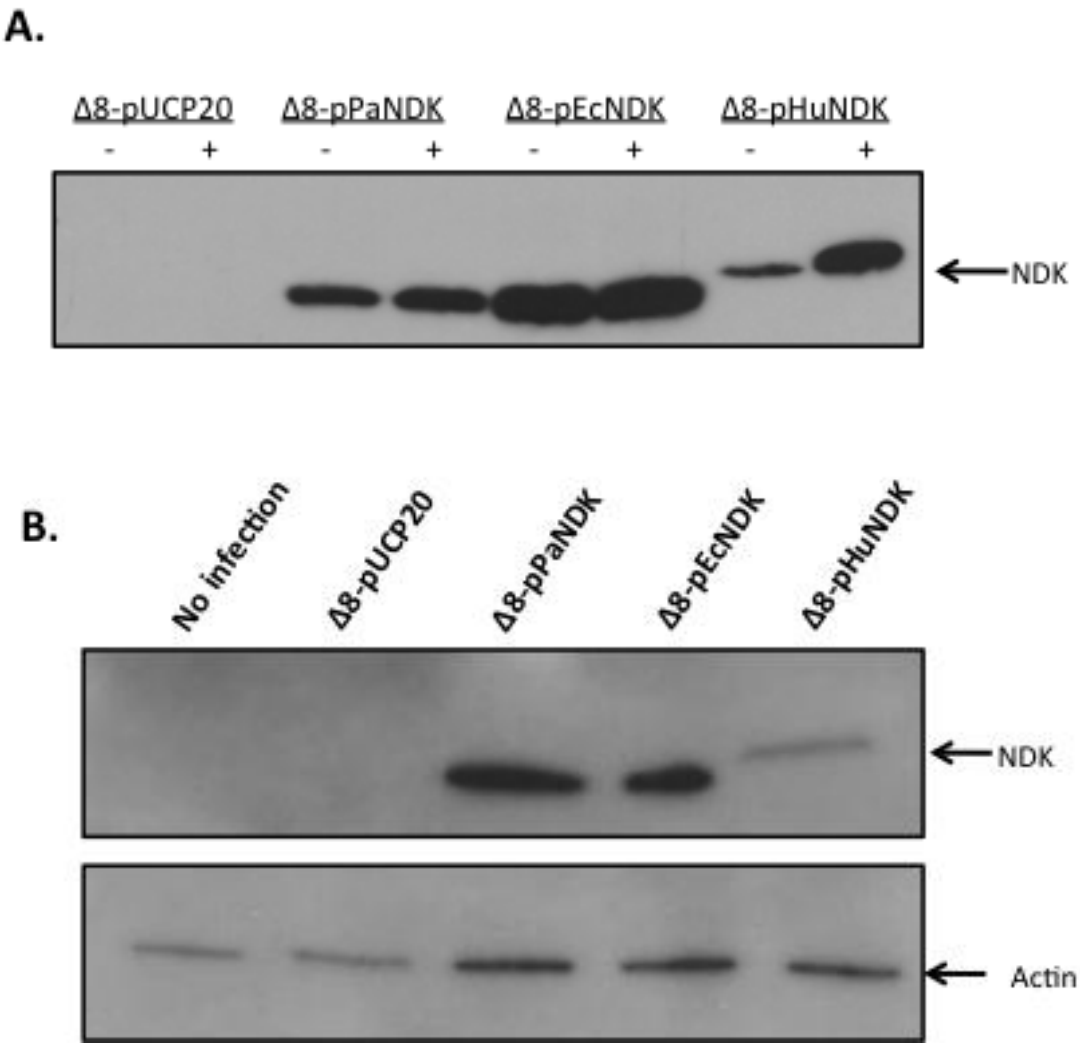

Figure 4-6. Secretion and injection of non-*P. aeruginosa* derived NDKs. (A) Bacteria were grown under type III inducing and non-inducing conditions and supernatants were subject to Western blotting with an antibody to the Flag tag. (B) HeLa cells were infected at an MOI 20 for 3 hours with the strains listed above. Cells were then collected, lysed, and subject to Western blotting with an anti-Flag antibody. Membranes were stripped and re-probed with an anti-actin antibody to serve as a loading control.

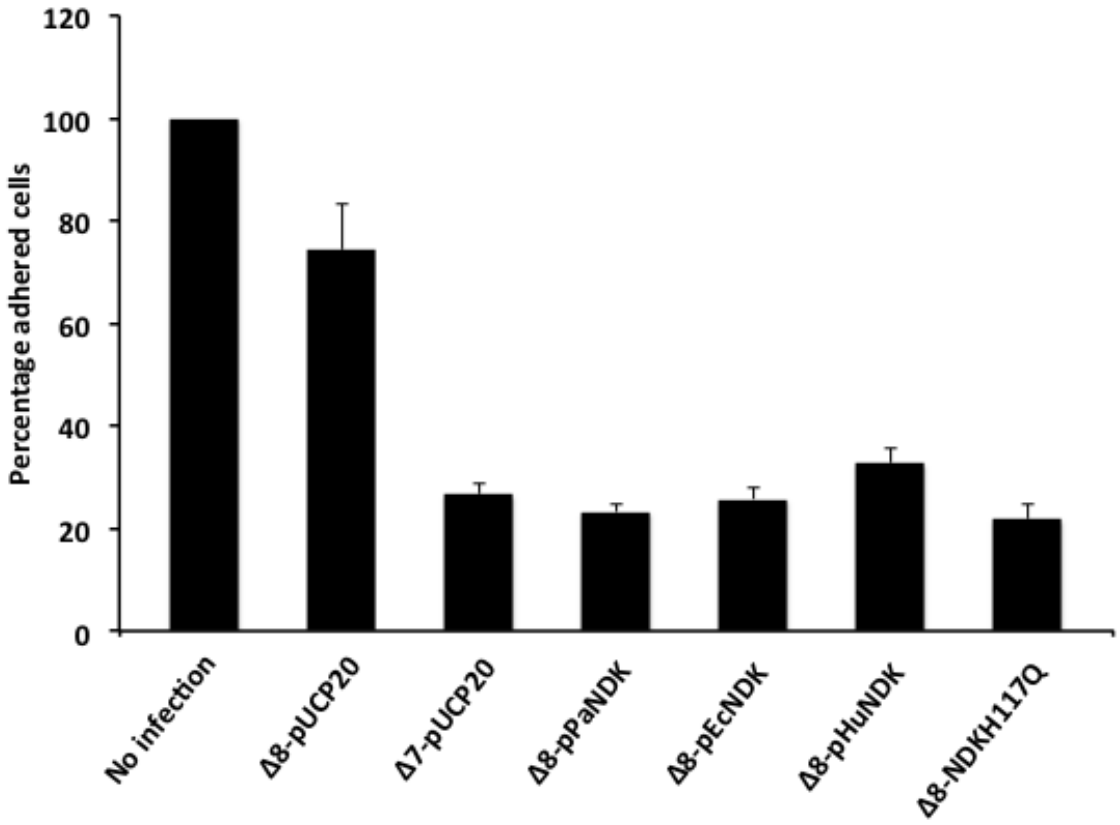

Figure 4-7. Cytotoxicity resulting from stains that possess NDK. HeLa cells were infected with bacterial strains for 5 hours at an MOI of 100. The bacteria were removed and cells were incubated for 16 hours in media containing antibiotics. Floating cells were removed and the remainder of adhered cells were collected and counted using a hemocytometer. Data is normalized to the no infection control.

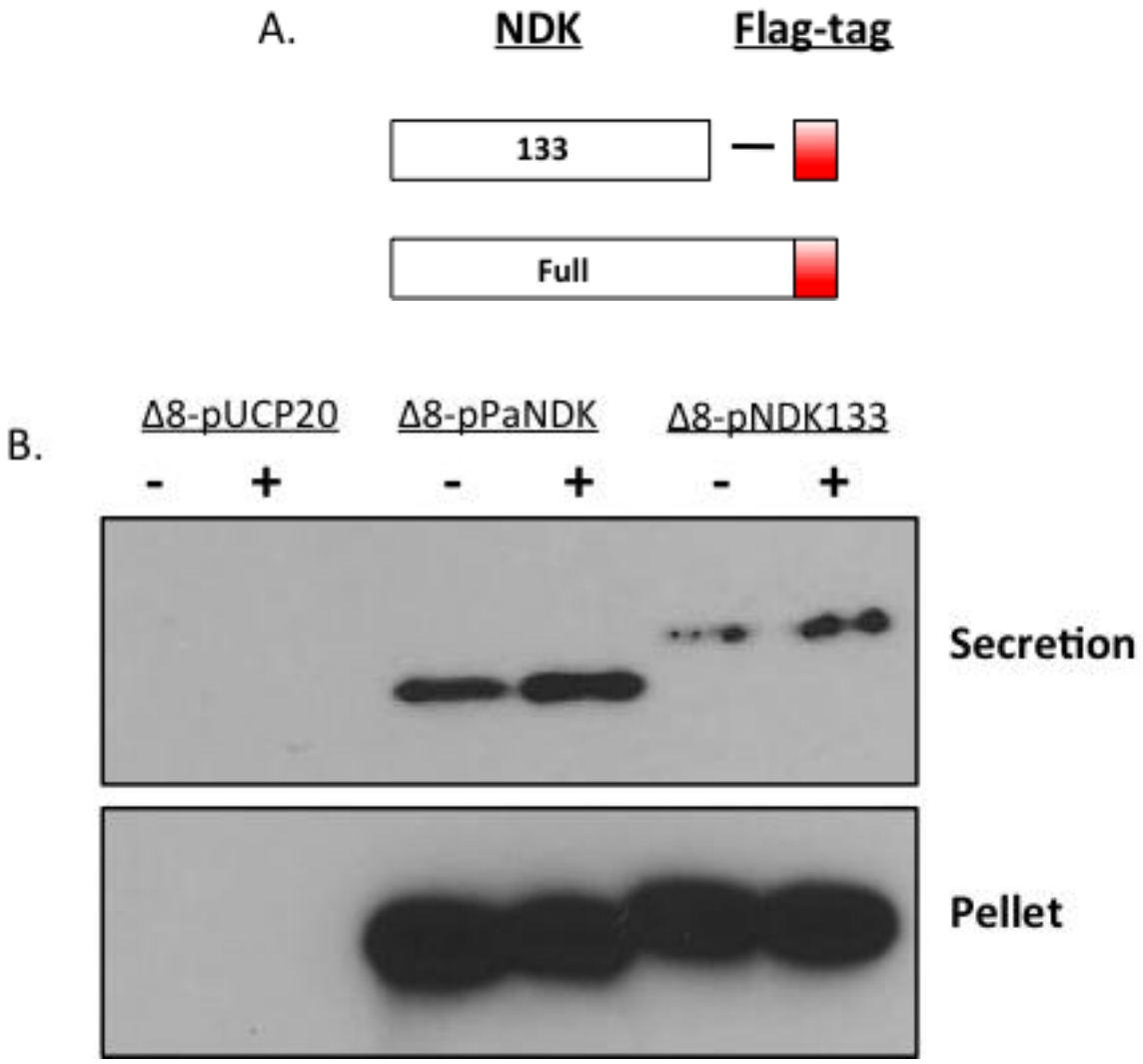

Figure 4-8. NDK lacking the T1SS signal sequence is not readily secreted. (A) Cartoon illustrating the NDK133 protein containing a C-terminal Flag tag. (B) Bacterial strains were grown in L broth or L broth containing 5mM EGTA for 3 hours. Supernatants were collected, run on an SDS-PAGE gel, and probed with an antibody against the Flag tag. Bottom panel shows whole bacterial cells that were lysed in protein sample loading buffer.

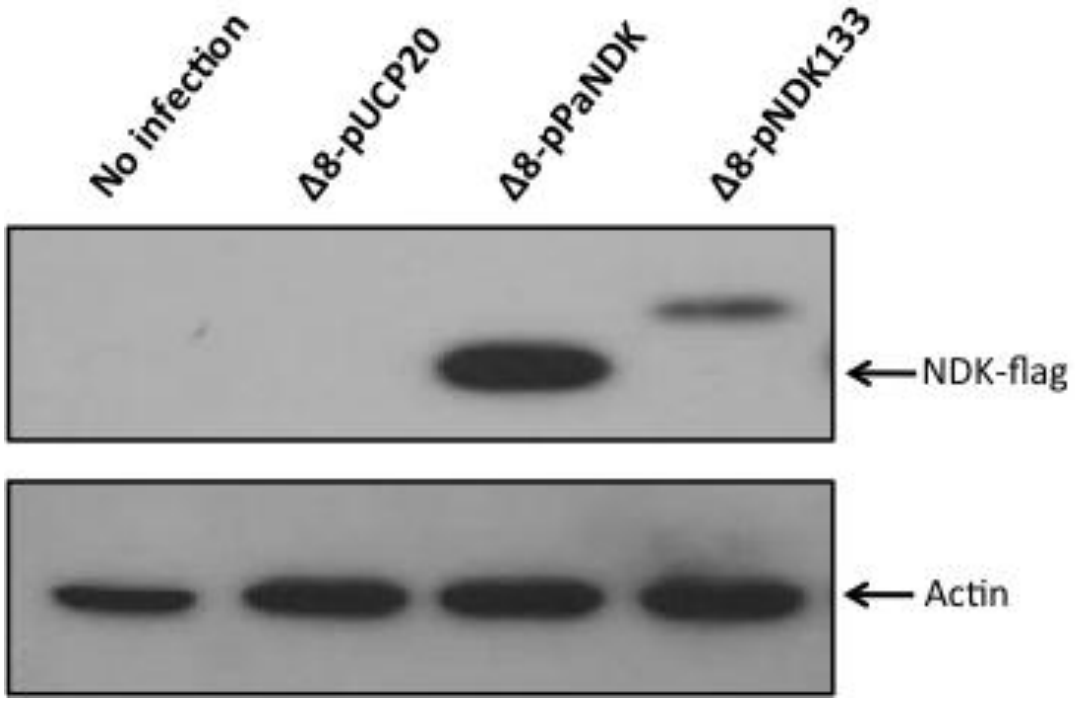

Figure 4-9. Type I secretion of NDK is necessary for protein injection. Bacteria were incubated with HeLa cells at an MOI 20 for 3 hours and cell lysates were probed with an antibody against the Flag-tag. Membrane was stripped and re-probed with actin as a loading control.

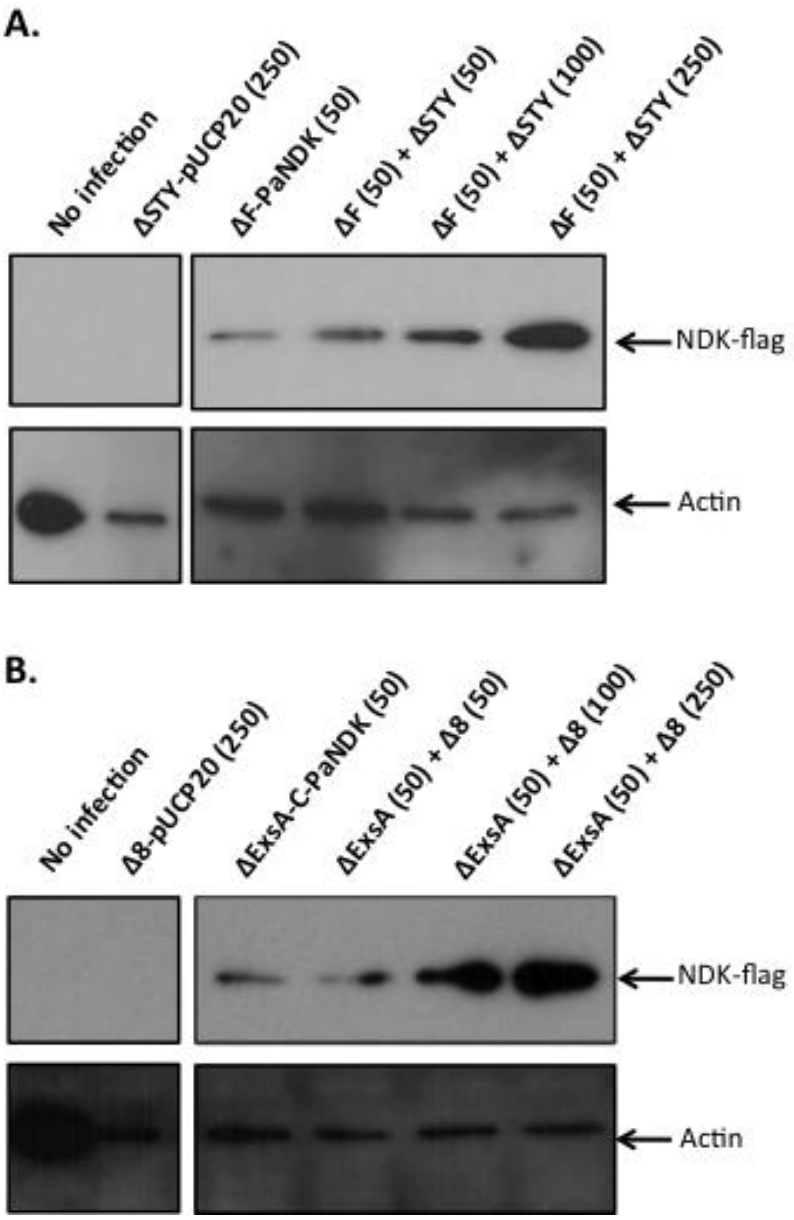

Figure 4-10. Type I secreted NDK can be injected via a functional T3SS. (A) A type III defective mutant containing flag-tagged NDK (ΔF-pPaNDK) was incubated with increasing MOIs of a strain lacking NDK, but containing a functional T3SS (ΔSTY-pUCP20). (B) Another type III defective strain containing flag-tagged NDK (ΔExsA-C-pUCP20) was incubated with increasing MOIs of a strain lacking NDK, but containing a functional T3SS (Δ8-pUCP20). Cells were collected, lysed, and subjected to Western blotting with an anti-Flag antibody. Membranes were stripped and re-probed with actin to serve as a loading control.

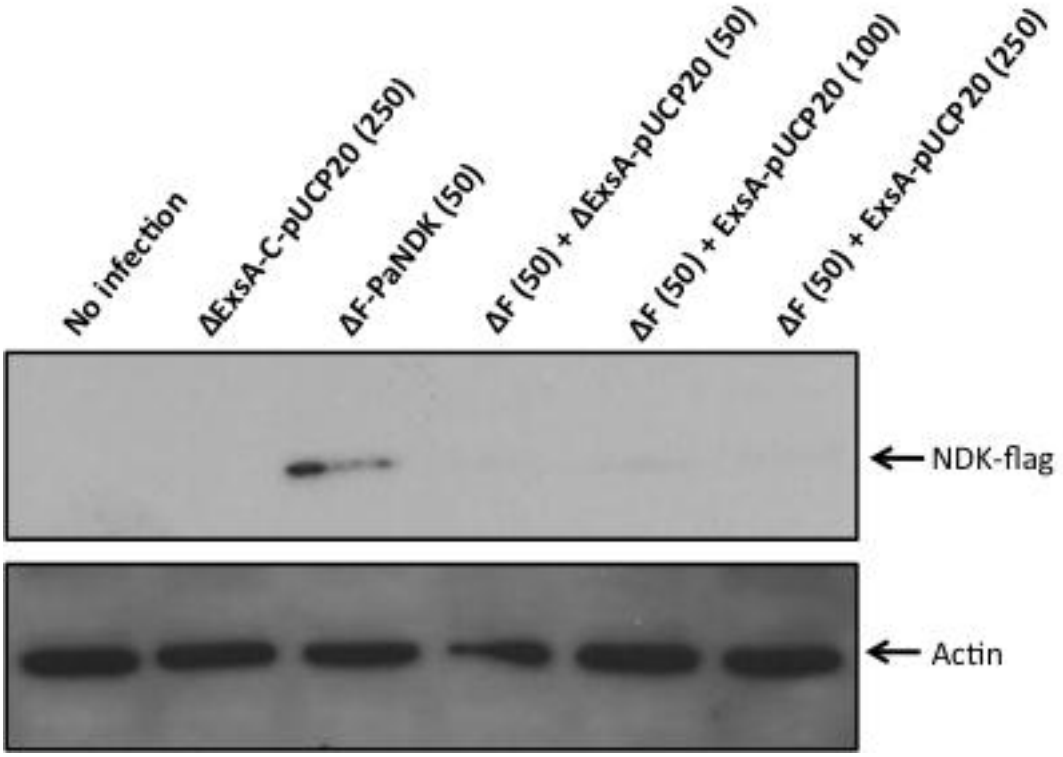

Figure 4-11. Type I secreted is not injected in the absence of a functional T3SS. A type III defective mutant containing flag-tagged NDK (ΔF-pPaNDK) was incubated with increasing MOIs of a strain lacking NDK and lacking a functional T3SS (ΔExsA-C-pUCP20). Following infection for 3 hours, HeLa cells were collected, lysed, and subject to Western blotting with an anti-Flag antibody. Membranes were re-probed with actin antibody to serve as a loading control.

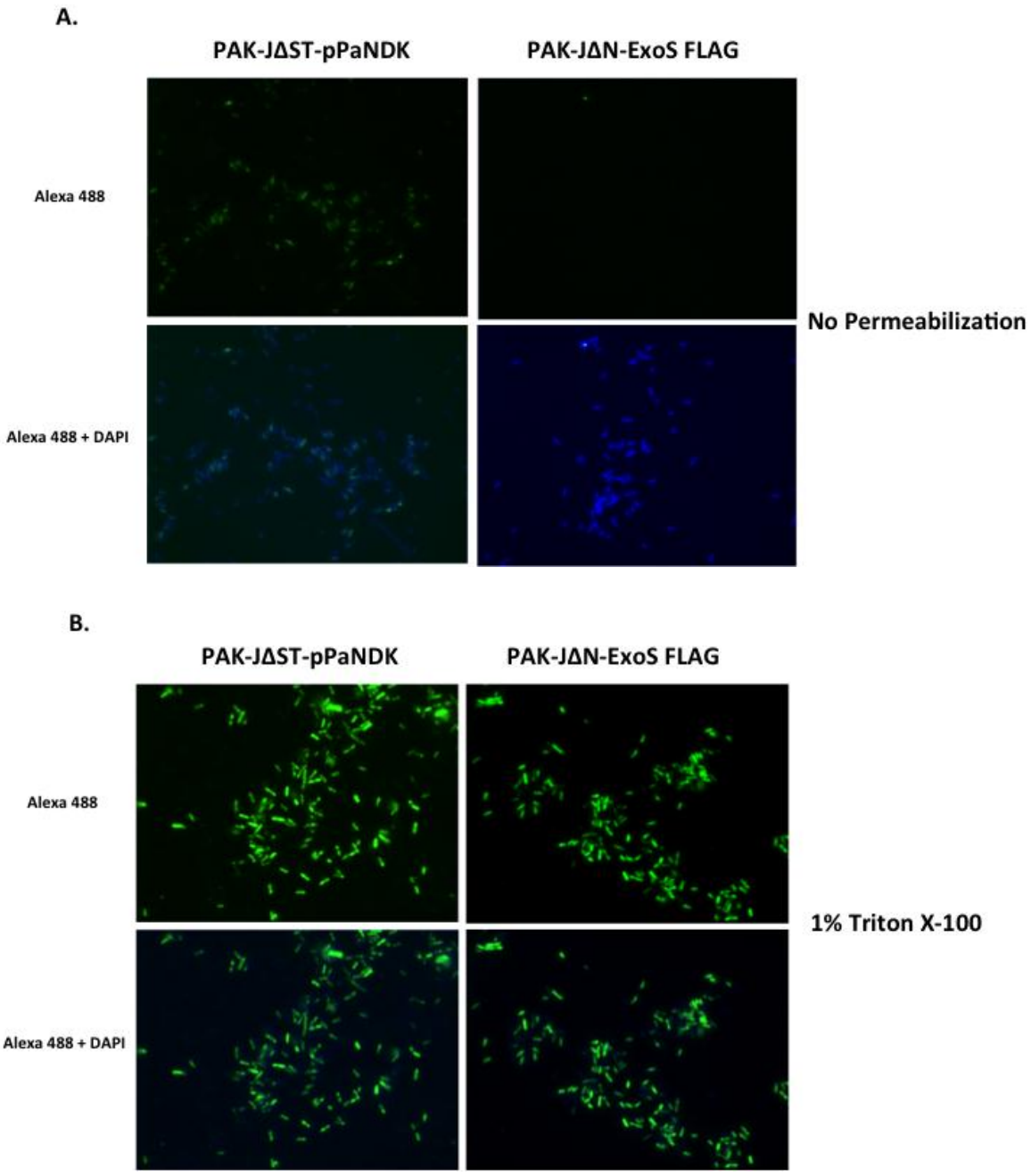

Figure 4-12. NDK is localized to the bacterial outer membrane. (A) Bacterial strains were sub cultured for 2 hours and placed on glass coverslips. Bacteria were then immunostained without permeabilization with an anti-Flag antibody and also treated with DAPI. (B) Strains were cultured under the same conditions above except they were permeabilized with 1% Trition X-100 before staining with anti-Flag antibody and DAPI.

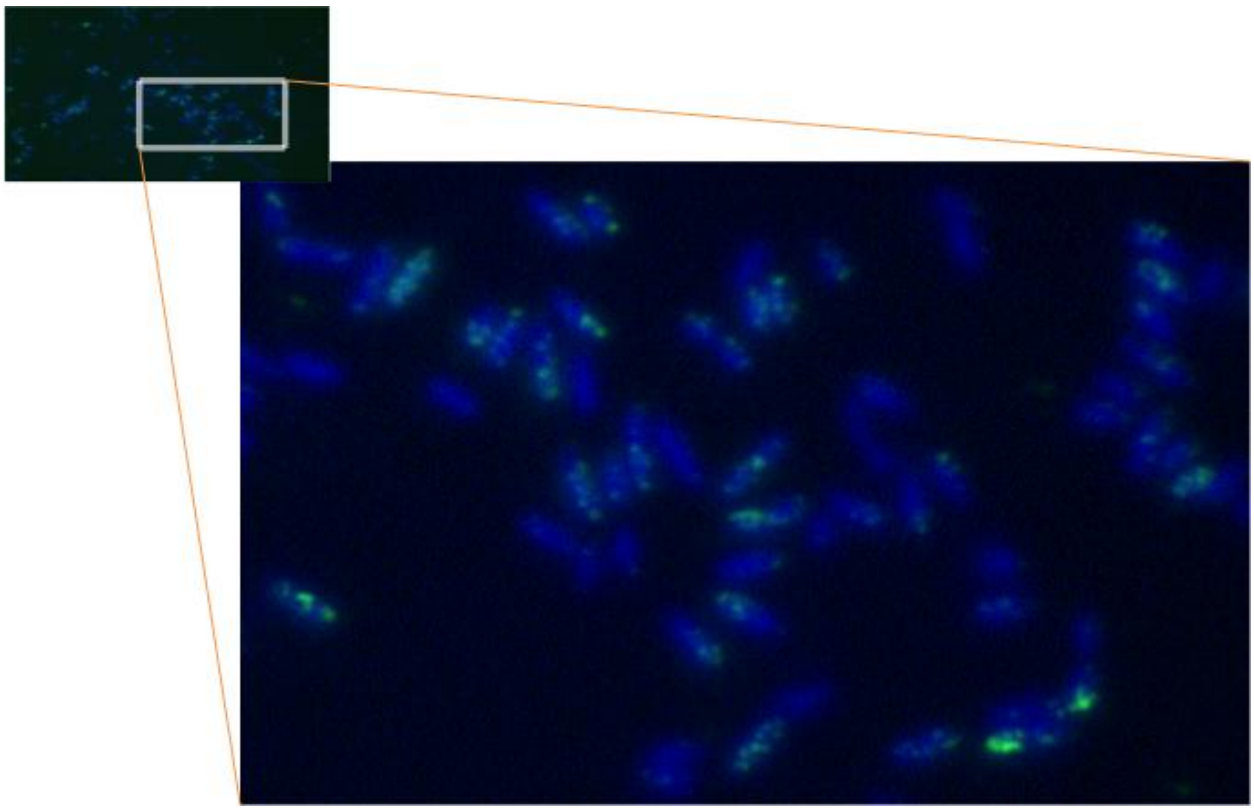

Figure 4-13. NDK on the bacterial surface. Bacterial cultures were prepared as listed in Fig. 4-12 and viewed under the 100X objective for a close up view. The bacteria were stained with DAPI and the green is NDK probed with an anti-Flag antibody. This shows NDK is located in patches around the bacteria outer surface

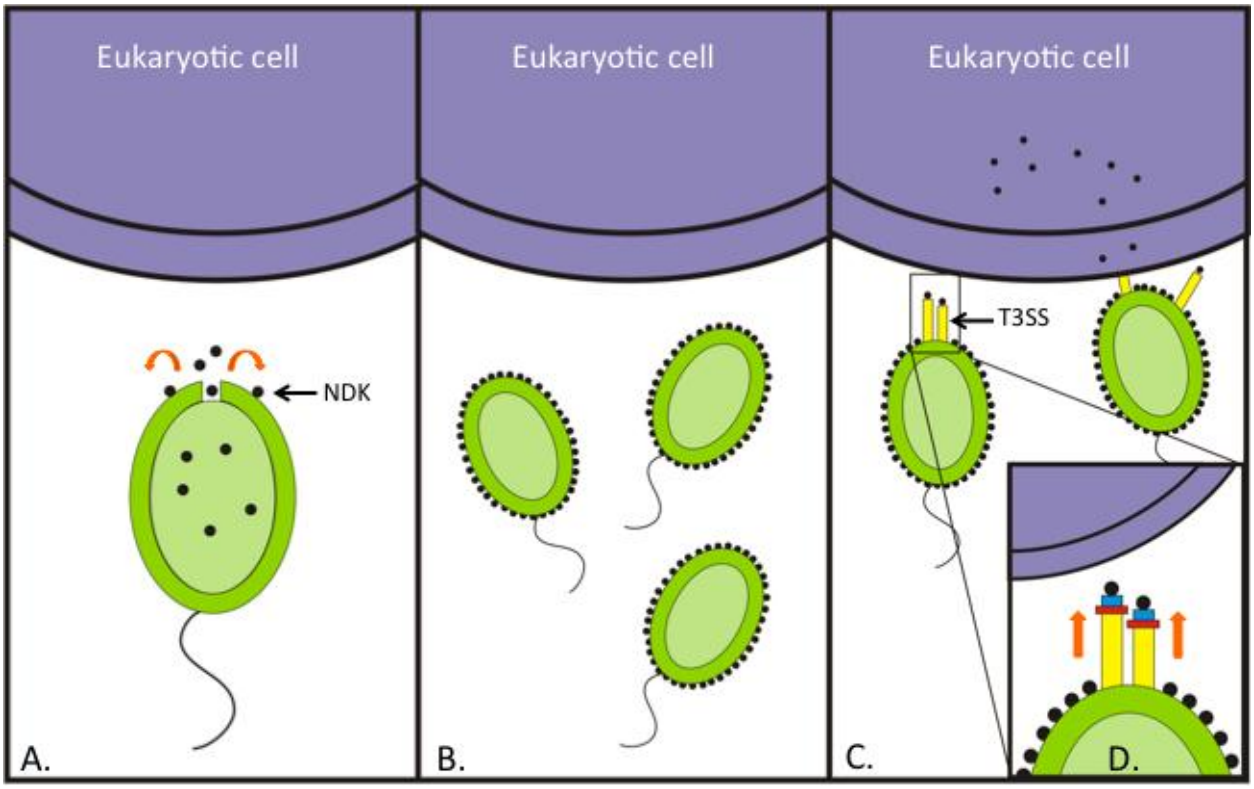

Figure 4-14. Proposed mechanism explaining NDK injection via the T3SS. (A) *P. aeruginosa* secretes NDK via its T1SS during the course of infection. (B) Secreted NDK binds to the bacterial outer surface and as bacteria approach the eukaryotic cell type III needles begin to form (C). (D) Magnified view showing that NDK binds to the type III needle as they are formed and is pushed by the needle into the host cell.

CHAPTER 5
THE USE OF EXOS AS A POTENTIAL ANTI-CANCER THERAPY

Background

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen that is responsible for causing diseases in immunocompromised individuals (62). Although *P. aeruginosa* possesses an arsenal of virulence factors, the T3SS is one of the most extensively studied. Toxicity is generated from the T3SS as a result of the injection of effector molecules from the bacteria, directly into the cytosol of the host cell (50). Currently, *P. aeruginosa* is known to possess four effectors: ExoS, ExoT, ExoU, and ExoY (38). Of these effectors, ExoS is the most studied and well characterized.

ExoS is a bi-functional toxin possessing C-terminal GAP and ADPRT domains (6). The GAP domain causes the rounding and lifting of infected cells due to its ability to disrupt cytoskelleton proteins such as Rho, Rac, and CDC42, while the ADPRT domain ADP-ribosylates multiple host cell target proteins (5, 28). Studies have shown that while the GAP domain of ExoS is able to cause cytotoxicity on its own, the activity of the ADPRT domain is dependent on the interaction with eukaryotic 14-3-3 proteins through a C-terminal 14-3-3 binding domain (182). Once activated, the APRT domain of ExoS is able to induce apoptosis in cells as early as three hours post infection (78, 81). ExoS is capable of generating toxicity in a broad population of cell types ranging from macrophages to cancer cells, with very few resistant cell lines being characterized, thereby making it an attractive candidate for an anti-cancer molecule (110, 131).

The use of bacteria and their products is not a novel concept in the area of cancer research (71). Current cancer treatments rely on the use of chemotherapeutic agents, which must be able to reach tumors via the bloodstream. However, due to the poor

vasculature of most tumors, drug delivery can be problematic (15, 132). Bacteria provide a unique solution to the drug delivery issue because they are able to adapt to their surroundings and can survive in the often hypo-oxygenic tumor environment. Live attenuated strains of *Salmonella typhimurium* have been developed that can specifically target tumors and effectively reduce their burden in mice (98, 185). However, the same reduction observed in mice has not been replicated in human clinical trials (157).

While the use of live attenuated strains is an emerging field in cancer research, there has been much focus on the use of bacterial toxins as anti-cancer agents. Two predominantly studied toxins are Exotoxin A (EA) from *P. aeruginosa,* and diptheria toxin from *Corynebacterium diptheriae*. Both toxins are mono-ADP ribosyltransferase enzymes that target elongation factor 2 (eFF2) and disrupt protein synthesis in eukaryotic cells ultimately leading to apoptosis (30, 169). These toxins are members of the AB family consisting of a cytotoxic domain (A domain) and binding or translocation domain (B domain) (13). During infection, these proteins are secreted by the bacteria, and through binding of the B domain with a eukaryotic cell receptor, are taken up by endocytosis (36). To target cancer cells, the binding domain of EA has been replaced with binding domains for cancer specific markers. Additionally, the binding domain has been replaced with an antibody to target cancer specific cells (83). There have been numerous phase 1 trials with recombinant forms of EA and some have yielded success leading to upcoming phase 2 trials (90, 173).

While EA is a promising therapeutic agent for the treatment of cancer, the use of recombinant toxins do have some drawbacks. Using antibody-conjugated toxins could lead to the production of antibodies against the drug itself. Also, there is the issue of

identifying novel cancer specific markers located on the tumor cells to ensure that drugs cause minimal damage to normal cells. Synthesizing a toxin that could not only target cancerous cells, but also specifically be activated in these cells, would be a great discovery for the field of cancer research.

Inactivation of the p53 transcription factor is found in nearly half of all reported cancer cases (58). Murine double minute 2 (MDM2) functions as a negative regulator of p53 by inhibiting the ability of p53 to bind to the transcriptional machinery of the host cell, or by ubiquintinating p53 and targeting it for degradation via the proteasome (26, 105). MDM2 is a protein upregulated in several forms of cancers such as lung, colon, and stomach, making it an attractive target for this study (155). MDM2 is known to inhibit p53 by binding to a 15 amino acid sequence located in the p53 transactivation domain, which is believed to induce a conformational change (92, 136). This binding between p53 and MDM2 is mostly composed of hydrophobic and electrostatic interactions, similar to the interaction between 14-3-3 and ExoS (106, 120). The p53 binding domain for MDM2 is comprised of an alpha-helix which fits into a binding cleft in MDM2 composed of alpha helices (92). ExoS is believed to be activated via a conformational change and based on the similarities it shares with MDM2-p53 interaction, we hypothesize that ExoS could be put under control of MDM2 by replacing the 14-3-3 binding domain of ExoS with the p53 binding domain of MDM2.

In this preliminary study, results are presented from experiments that attempt to put ExoS under control of MDM2, a protein that is over expressed in about 10% of all reported cancer cases (156). The goal was to demonstrate that ExoS could induce cytotoxicity in an MDM2 dependent manner, thereby providing a proof of principle

demonstrating that a toxin could be placed under control of a protein highly expressed in certain forms of cancer. While the results of these experiments were contrary to our initial hypothesis, they do reveal some interesting and novel insights into the role 14-3-3 proteins play in the activation of ExoS.

Materials and Methods

Construction of ExoS Fusion Proteins

The ExoS-P53 fusion proteins were synthesized using the primers listed in Table 2-3. The p53, PDIQ, and 1E6N binding sequences were incorporated into the primers. The template DNA used to synthesize these constructs contained an amino acid substitution at position 146 of ExoS which renders the GAP domain catalytically inactive. Following PCR, the fusion proteins were cloned into the pUCP20 vector with *EcoRI* and *HindIII* restriction sites. The ExoSΔ14-3-3 was created with the primers listed in Table 2-3 and was cloned into pUCP20 with *EcoRI* and *HindIII* restriction sites. All of the constructs were confirmed by restriction enzyme digestions and DNA sequencing.

Secretion Assay for ExoS-P53 Fusion Proteins

Bacterial strains were grown overnight in 1.0 ml of L broth containing carbenicillin at 37°C. Overnight cultures were then inoculated at 5% into fresh L broth containing antibiotics for non-type III inducing conditions and L broth plus antibiotics and 5mM EGTA for type III inducing conditions. *P. aeruginosa* strains were grown in a shaking incubator at 37°C for three hours, after which bacteria were collected and spun down at 20,000xg. Bacterial supernatants were collected, mixed with equal volumes of protein sample buffer and boiled for 10 minutes before subjecting to SDS PAGE analysis. Supernatants were run on SDS-PAGE gels and subject to Western blotting with an antibody recognizing ExoS.

Protein Injection Assay

Cells were seeded into 6 well plates at approximately 70 % confluency (8.4×10^5 cells) in medium containing antibiotic the night before infection. Two hours prior to infection, cells were washed twice in 1X PBS and replaced with medium containing no antibiotics. Bacterial strains were grown in L broth supplemented with carbenicillin at 37˚C until the OD_{600} reached 0.8. For an MOI of 50, 5×10^7 CFU per ml of bacteria were incubated with eukaryotic cells for the indicated amount of time. Following infection, bacteria were washed away and cells were harvested by scraping. Cells were spun down at 500xg for 5 minutes and then washed with PBS for 3 times. Cells were lysed by incubating in 0.25% Triton X-100 in PBS for 10 minutes on ice. Following lysis, cells were centrifuged at 20,000xg for 2 minutes. The supernatants were collected and mixed with 50µl of protein sample buffer and boiled for 10 minutes. Lysates were run on SDS-PAGE gels and subject to Western blotting with an antibody recognizing ExoS.

Cell Viability Assay

The assay used to measure cell viability was the Invitrogen LIVE/DEAD viability/cytotoxicity kit for mammalian cells (catalogue number L-3224). Cells were infected for 3 hours at an MOI of 20 with the indicated bacterial strains. Bacteria were cleared by washing cells 3x with PBS and then cells were collected by incubation with 0.25% trypsin for 5 minutes. The cells were then pelleted by centrifugation at 400xg for 5 minutes. The pellet was suspended in 150µl of the live/dead staining solution, which was composed of 2µM calcein (component A) and 4µM EthD-1 (component B), for 45 minutes at room temperature. Samples were then mixed and 10µl of cells were placed on a glass coverslip and viewed under a fluorescent microscope.

Ras Modification Assay

HeLa cells were seeded into 6 well plates at approximately 70% confluency (8.4 x 10^5 cells) in medium containing antibiotic the night before infection. Two hours prior to infection, cells were washed twice in 1X PBS and replaced with medium containing no antibiotics. Bacterial strains were grown in L broth supplemented with carbenicillin at 37˚C until the OD_{600} reached 0.8. For an MOI of 50, 5 x 10^7 CFU per ml of bacteria were incubated with HeLa cells for the indicated amount of time. Following infection, bacteria were removed by washing cells 3x in PBS and cells were collected by scraping. The cells were pelleted by centrifugation at 400xg for 5 minutes and then lysed by adding 100µl of protein sample buffer. Samples were boiled for 10 minutes and then subjected to Western blotting with an antibody that recognizes all 3 isoforms of Ras (BD Transduction Laboratories anti-Ras 610001).

Results

Generation of ExoS-P53 Fusion Proteins

ExoS requires binding to eukaryotic 14-3-3 proteins via its C-terminal 14-3-3 binding domain in order to elicit a cytotoxic response in host cells (48). While ExoS is a well-characterized protein, the role in which 14-3-3 proteins play in facilitating the toxic response is still poorly understood. In an effort to put ExoS under control of a cancer specific protein, we made several ExoS fusion proteins in which the 14-3-3 binding domain had been replaced with various forms of the p53 binding domain for MDM2 (Figure 5-1). The p53 binding domain for MDM2 has been well characterized and is similar in amino acid length to the 14-3-3 binding domain of ExoS (120). The interaction of MDM2 with the p53 transactivation domain is composed of mainly hydrophobic and electrostatic interactions, similar to the interactions between ExoS and 14-3-3 (92). An

ExoS fusion protein was synthesized so that the p53 binding domain would be in-frame with ExoS and replace the C-terminal 14-3-3 binding domain (Figure 5-1). Additionally, we generated a construct containing a mutated sequence of the p53 binding domain, known as PDIQ, which has been shown to have five times greater affinity for MDM2 (126). As a control construct, ExoS was fused to a p53 binding domain which had been mutated so that it was no longer capable of interacting with MDM2 (ExoS-1E6N) (126). To ensure that any cytotoxic effects observed were from the ADPRT domain, the GAP domain in all of the constructs was rendered catalytically via an amino acid substitution at position 146 (56).

Secretion and Injection of ExoS-P53 Fusion Proteins via the T3SS

Following construction, ExoS-P53 fusion genes were introduced into the PAK-JΔ*STY* background and assayed for secretion. Strains were grown under type III inducing conditions for three hours and supernatants were collected and subjected to Western blotting with an antibody against ExoS. Figure 5-2A shows that all of the ExoS-P53 fusion constructs were secreted at levels similar to the wild type ExoS (pHW0029 and PAK-J lanes). To demonstrate that the GAP domain does not play a role in the secretion process, a plasmid expressing ExoS with a mutated GAP domain (pExoSΔGAP) was tested, which also showed similar secretion levels to the wild-type protein.

The same strains were also tested for their ability to inject the fusion proteins into mammalian cells. HeLa cells were incubated with the bacterial strains for three hours at an MOI 20. The bacteria were cleared, and the cell lysates were subject to Western blotting. As illustrated in Figure 5-2B, all of the ExoS fusion proteins were translocated at similar amounts compared to the native ExoS. Taken together, these results show

that altering the 14-3-3 binding domain of ExoS does not interfere with ability of the protein to be secreted or injected into eukaryotic cells.

ExoS-P53 Fusion Proteins Show Reduced Cytotoxicity

In order to determine if the ExoS-P53 fusion constructs were able to elicit a cytotoxic response in an MDM2 dependent fashion, we employed the use of two MEF cell lines, one deficient in p53 and the other deficient in $p53^{-/-}$, $MDM2^{-/-}$, $MDMX^{-/-}$. Deletion of MDM2 alone is embryonic lethal however, in combination with deletion of p53, embryos survive (79, 111). Both cell lines were incubated with bacterial strains expressing the ExoS-P53 fusion proteins for three hours at an MOI of 20. Following infection, bacteria were cleared and the number of viable cells was determined. Figure 5-3 shows that strains expressing the ExoS-P53 fusion proteins failed to elicit a cytotoxic response in either cell line as robust as wild-type ExoS (pHW0029). Additionally, all of the ExoS-P53 constructs appeared to cause similar levels of toxicity that appeared to be independent of MDM2 expression. While these constructs were not as toxic as native ExoS, they did appear to generate slightly elevated levels of cell death compared with infection from a strain expressing a form of ExoS that has both cytotoxic domains inactivated (pHW0224).

To confirm these results, we carried out similar infections in the $p53^{-/-}$, $MDM2^{-/-}$, and $MDMX^{-/-}$ cell line and compared them to infections in a cell line that overexpresses MDM2 via a eukaryotic expression plasmid (H1299-HDM2 cells). As observed in the previous infections, all of the ExoS-P53 constructs failed to generate a cytotoxic response as great as ExoS (Figure 5-4). They did however, cause more toxicity than infection with the ExoS mutant although it appeared independent of MDM2 (Figure 5-2). Taken together, these results demonstrate that while the ExoS-P53 fusion proteins lack

the potency of the wild-type ExoS, they do appear to generate a marginal toxic response independent of the presence of MDM2.

ExoS-P53 Fusion Protein ADP-Ribosylate Ras

The ADPRT domain of ExoS has been shown to induce apoptosis in cells by ADP-ribosylating the cell signaling protein Ras (86). However, toxicity occurs only after ExoS is activated by its eukaryotic co-factor, 14-3-3. Infections with *P. aeruginosa* strains harboring mutations in the ADPRT or 14-3-3 binding domain of ExoS prevent the modification of Ras, resulting in dramatically reduced cytotoxicity (69). Although cytotoxicity assays suggested the ExoS-P53 fusion constructs were not responding to MDM2, it is possible that MDM2 is not as efficient at activating ExoS as the native activator, 14-3-3. We therefore wanted to examine if the low level toxicity produced by the ExoS-P53 fusion proteins was a result of a functional ADPRT domain. To test this, strains containing the ExoS-P53 fusion proteins were incubated with HeLa cells for three hours at an MOI of 20. Lysates from infected cells were run on an SDS-PAGE and probed with an antibody against Ras. Previous studies have demonstrated that the molecular weight of Ras is increased by approximately 2kD following ADP-ribosylation from ExoS, making a convenient assays system for testing the functionality of the ADPRT domain (109). Infection with PAK-J, PAK-JΔ*STY*/pHW0029, and PAK-JΔ*STY*/pExoSRK, resulted in the modification of Ras, whereas infection with ExoS containing an inactive ADPRT did not (Figure 5-5). All of the ExoS-P53 proteins displayed the ability to modify Ras suggesting they possess a functional ADPRT. This phenomenon was dependent on a functional T3SS, as infection with a type III defective strain expressing the ExoS-PDIQ fusion protein (PAK-JΔ*D*/pExoSPDIQ) was unable to modify Ras (Figure 5-5).

Interestingly, a control construct lacking the GAP domain and 14-3-3 binding domain (pExoSΔ14-3-3) was also able to modify Ras (Figures 5-1, 5-5). It has been well documented that perturbing the 14-3-3 binding results in a form of ExoS that is unable to ADP-ribosylate Ras however, no studies have been conducted in which the entire binding site was removed. To ensure this observation was a result of the ExoSΔ14-3-3 protein, we tested the ability of this protein to be secreted and injected into cells. Results from those experiments confirm that ExoSΔ14-3-3 was secreted and injected at levels comparable to the wild-type ExoS (Figures 5-2,5-3). Taken together, these results demonstrate that the ExoS-P53 fusion proteins do possess a functional ADPRT domain even in the absence of 14-3-3 binding. Additionally, they show that a form of ExoS lacking the 14-3-3 binding domain is still able to modify Ras.

ExoSΔ14-3-3 Modifies Ras at a Slower Rate

Majority of the toxicity associated with ExoS results from the ADP-ribosylation of Ras (67). Interestingly, ExoSΔ14-3-3 was able to ADP-ribosylate Ras like the wild-type form, yet it does not generate as robust of a cytotoxic effect. ExoS is able to induce toxicity in eukaryotic cells within as little as 30 minutes post infection (81). Based on this, we examined the modification of Ras in HeLa cells at various time points post infection with PAK-JΔ*STY*/pExoSRK or PAK-JΔ*STY*/pExoSΔ14-3-3. As seen in Figure 5-6, PAK-JΔ*STY*/pExoSRK is able to completely modify Ras by 30 minutes post infection. Although ExoSΔ14-3-3 is able to cause some modification of Ras by 30 minutes, it is not to the extent by ExoSRK until 120 minutes post infection. Based on these observations, it appears that ExoSΔ14-3-3 modifies Ras at a slower rate, which might account, at least in part, for its reduction in cytotoxicity.

Discussion

The overall goal of this preliminary study was to demonstrate that the ADPRT domain of ExoS could be placed under control of a cancer specific protein. In an effort to accomplish this, the 14-3-3 binding domain of ExoS was replaced with the p53 binding domain for MDM2. Two variations of this construct were created, with one containing a mutated p53 domain which possesses five times greater affinity for MDM2 (ExoS-PDIQ) and the other containing a mutated sequence which can no longer bind MDM2 (1E6N) (Figure 5-1). These constructs were readily secreted and injected into eukaryotic cells by *P. aeruginosa* demonstrating that disruption of the 14-3-3 binding domain does not alter these processes (Figure 5-2). Our prediction was that both the ExoS-p53 and ExoS-PDIQ constructs would induce cytotoxicity at levels comparable to wild-type ExoS in an MDM2 dependent fashion, whereas the ExoS-1E6N would not due to its inability to interact with MDM2 (126). Infection assays in cells lacking MDM2 and cells expressing MDM2, demonstrate that the ExoS fusion proteins were greatly attenuated in cytotoxicity compared to the wild-type ExoS (Figure 5-3). These results also suggest that the toxicity observed from the ExoS-P53 fusion proteins was not likely to be MDM2 dependent. These results were also confirmed by doing similar infections between an MDM2 null cell line, and a cell line overexpressing MDM2 from a eukaryotic expression plasmid (Figure 5-4).

While we failed to see the results we predicted, we did observe that the ExoS-P53 fusions caused slightly higher toxicity in cells than infection with a *P. aeruginosa* stain expressing a GAP and ADPRT deficient ExoS (Figures 5-3, 5-4). Since ExoS elicits apoptosis in cells by ADP-ribosylating the small signaling molecule Ras, we conducted experiments to determine if the ExoS-P53 fusion proteins had functional ADPRT

domains. Interestingly, infection experiments in HeLa cells revealed the all three ExoS-P53 proteins were able to modify Ras as efficiently as wild-type ExoS, however these proteins failed to induce the same level of toxicity in the course of a three hour infection (Figure 5-5).

Another striking observation made was that our control construct lacking the 14-3-3 binding domain was also able to modify Ras. As is the case with the ExoS-P53 fusions, ExoSΔ14-3-3 is less toxic than the wild-type form. This observation is interesting because it is the first time that anyone has demonstrated that removal of the 14-3-3 binding domain from the full length ExoS protein is still able to ADP-ribosylate Ras. Henriksson et al have demonstrated that 14-3-3 proteins are required for the inhibition of Ras by ExoS (69). In their study, an ExoS protein comprised of amino acids 88-453 was able to modify Ras, whereas a from lacking the 14-3-3 binding (ExoS 88-426) domain was not. However, a study by Pederson et al, demonstrated that ExoS lacking the MLD, but containing the 14-3-3 binding domain, was unable to modify either membrane bound or cytosolic forms of Ras (123). The MLD of ExoS is located from amino acids 52-72, so perhaps the reason that Henriksson et al did not observe ADP-ribosylation of Ras with their ExoS 88-426 protein was because it lacked the MLD (69). Taken together with the results from this work, it appears as though ExoS might need both the 14-3-3 binding domain and the MLD in order to cause maximum toxicity. This could explain why toxicity assays from Henriksson et al using ExoS 88-453 showed lower levels of cytotoxicity than similar assays from our laboratory (78).

The exact role of 14-3-3 proteins play in activating ExoS is still unclear. Currently, there are three classifications for the actions of 14-3-3 proteins binding to target

proteins: i) induction of a conformational change in the target protein, ii) occlusion of a sequence specific regions of the target protein, and iii) function as a scaffold to promote binding between two proteins (19, 161). The current hypothesis is that 14-3-3 proteins bind to ExoS and induce a conformational change making the ADPRT active (120). However, evidence presented here suggests the 14-3-3 proteins might be acting as scaffold to facilitate interactions with Ras. Time course assays revealed that ExoSΔ14-3-3 not only ADP-ribosylates Ras, but that it takes 90 minutes longer to reach the same levels of modification that wild-type ExoS does in 30 minutes (Figure 5-6). Perhaps only the kinetics of the toxicity is changed in the ExoSΔ14-3-3 construct, given that it merely takes longer to reach the same levels as the native form since it is missing a protein that facilitates binding to the target molecule.

The question still remains as to how the ExoS-P53 fusion proteins are able to modify Ras. Perhaps MDM2 is playing a role and the assays preformed thus far are not sensitive to detect a difference. Although ExoS-1E6N was predicted to have low toxicity, it displayed levels similar to both ExoS-P53 and ExoS-PDIQ. We cannot rule out the possibility that other cellular proteins are binding to these constructs and activating them. Also, based on the observations from the ExoSΔ14-3-3 protein, we cannot rule out the possibility that the ADPRT domain is constitutively active, yet needs a protein to promote binding to its target Ras. This could explain why the ExoS-P53 fusion proteins were able to modify Ras. More studies are needed to resolve the role of 14-3-3 proteins in the activation of ExoS. Once there is a firm understanding, it might be possible to harness ExoS to promote efficient killing of cancerous cells.

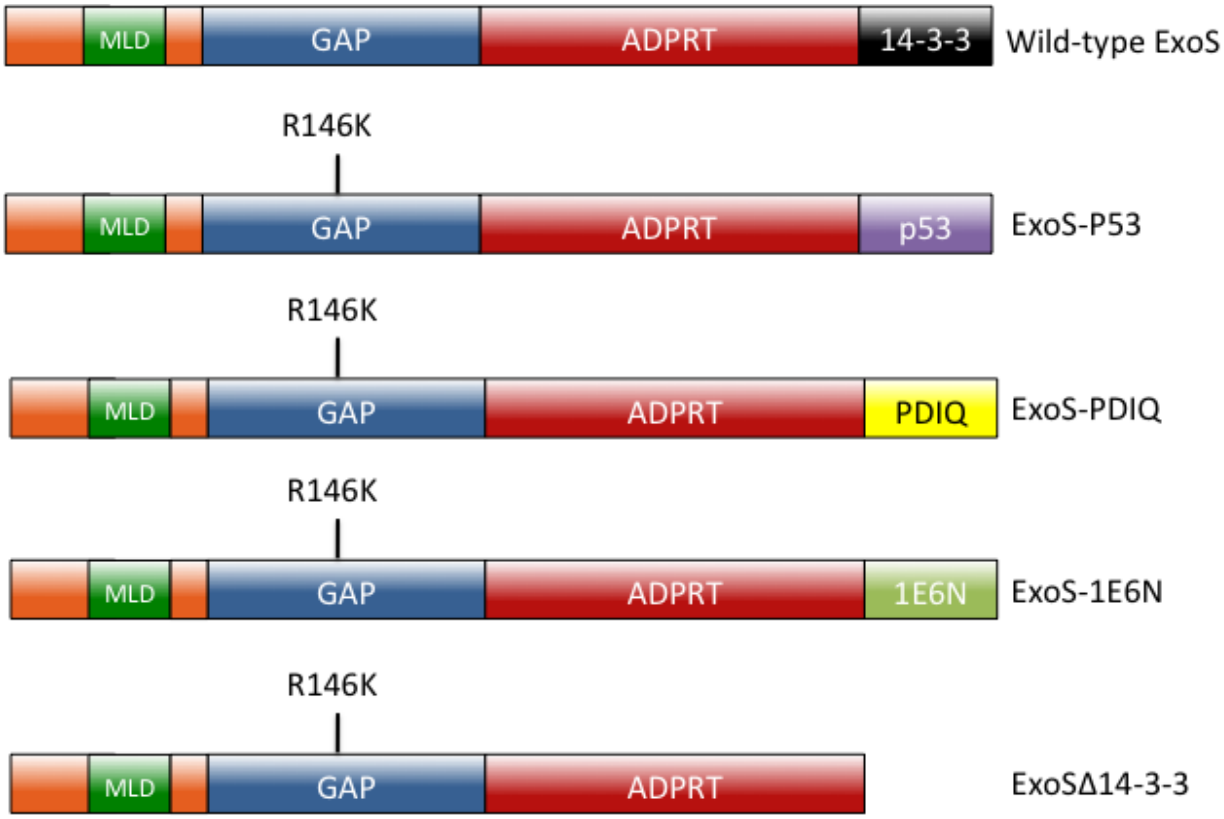

Figure 5-1. Diagram illustrating ExoS-P53 fusion proteins. ExoS-P53 fusions were synthesized to contain a p53 binding sequence in place of the wild-type ExoS 14-3-3 binding domain. ExoS-P53 has the native p53 binding sequence for MDM2, while PDIQ has a 5 time greater affinity binding sequence for MDM2. The 1E6N sequence is mutated to prevent p53 binding with MDM2. Additionally we made a control construct lacking the 14-3-3 binding domain of ExoS. All synthesized constructs contained a mutated GAP domain to ensure any toxicity seen was resulting from the ADPRT domain.

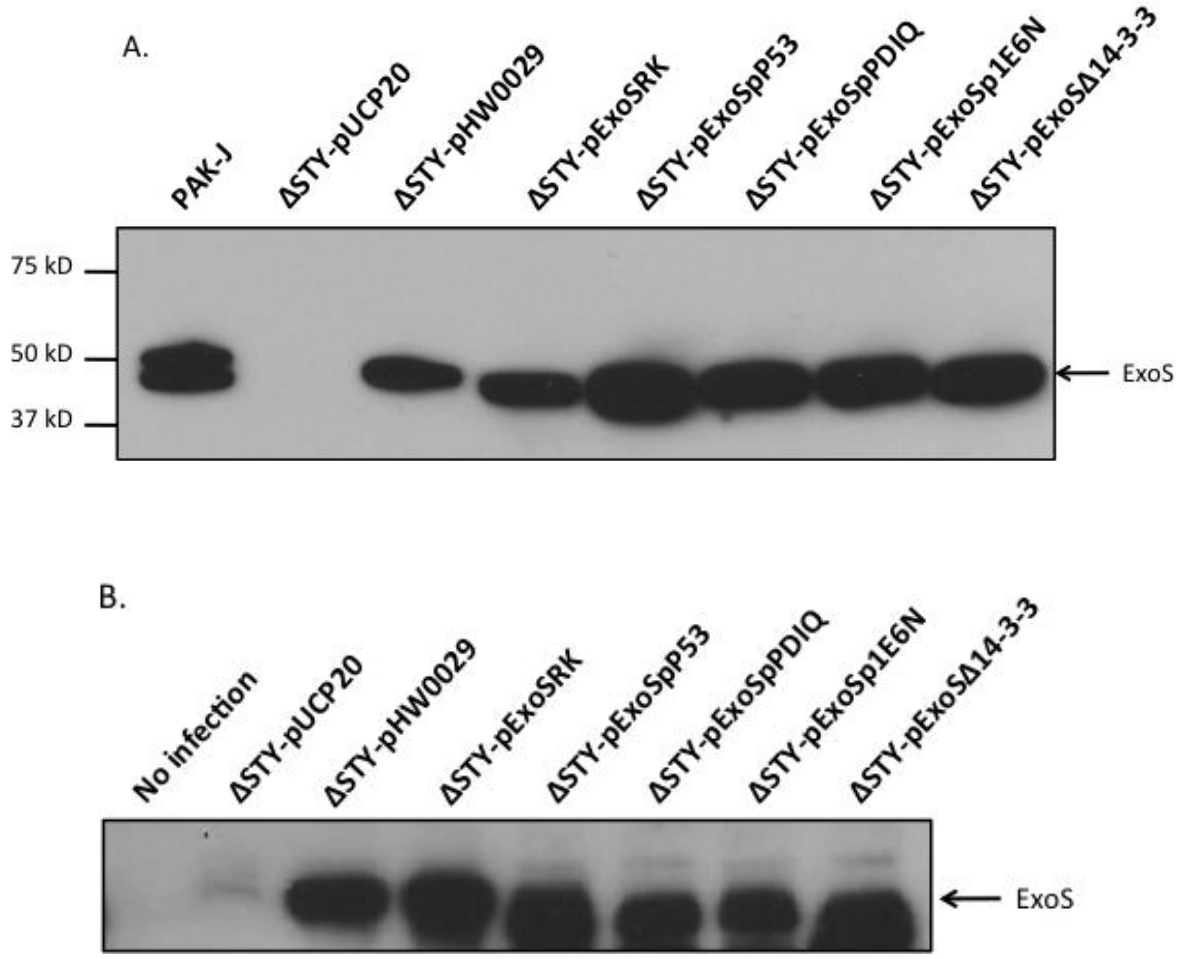

Figure 5-2. Secretion and injection of the ExoS-P53 fusion proteins. (A) Secretion assay results from strains possessing the ExoS-P53 fusion constructs. Bacteria were grown for 3 hours under type III inducing conditions and supernatants were collected and probed with an antibody against ExoS. (B) Injection of ExoS-P53 proteins in HeLa cells. Cells were infected for 3 hours at an MOI of 20 and then collected and lysed. Lysates were run on a SDS-PAGE gel and probed with an anti-ExoS antibody.

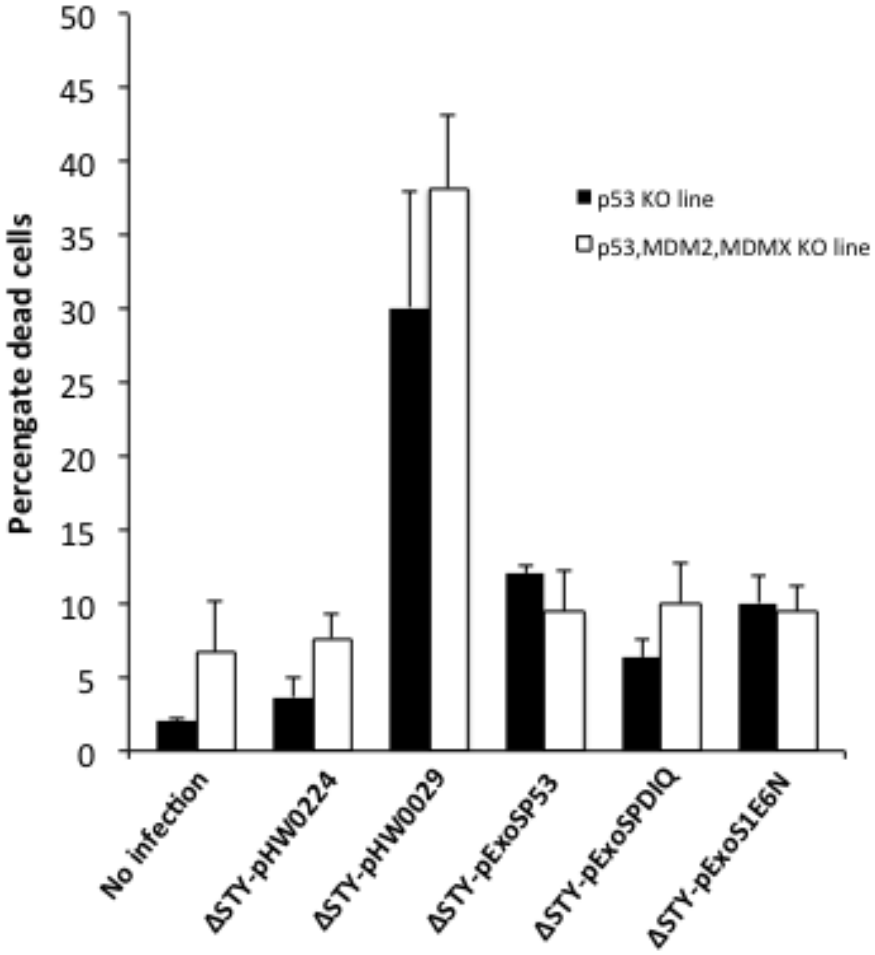

Figure 5-3. Cytotoxicity of ExoS-P53 fusion proteins. A p53 knockout cell line and a p53, MDM2, and MDM2 knockout cell line were infected with the indicated bacterial strains for 3 hours at an MOI of 20. Following infection, bacteria were cleared and the cells were stained for live and dead cells using a live/dead cell viability assay. The number of live versus dead cells were counted under a fluorescent microscope using a hemocytometer.

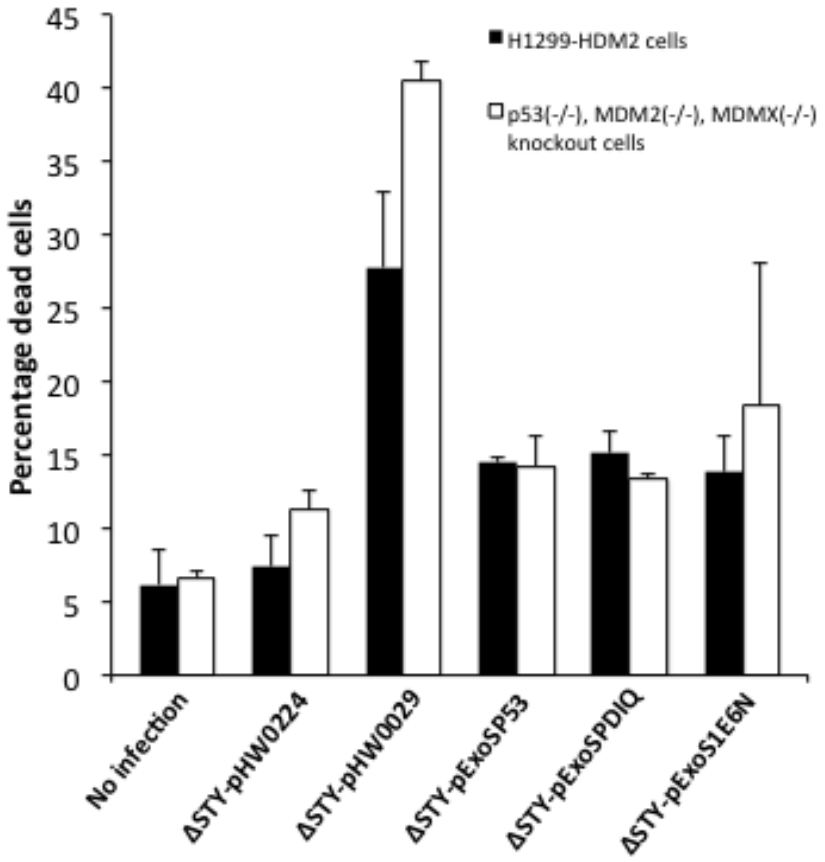

Figure 5-4. ExoS-P53 fusion protein cytotoxicity in MDM2 overexpressing cells. The p53, MDM2, and MDMX knockout cell line and the H1299-HDM2 cell line, which overexpresses MDM2, were infected with the indicated bacterial strains at an MOI of 20 for 3 hours. Following infection, bacteria were cleared and the cells were stained for live and dead cells using a live/dead cell viability assay. The number of live versus dead cells were counted under a fluorescent microscope using a hemocytometer.

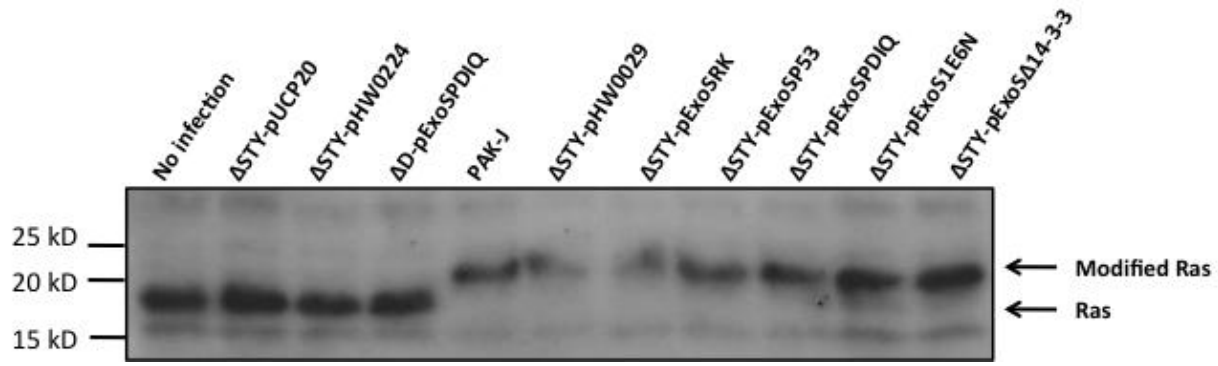

Figure 5-5. Modification of Ras protein by the ExoS-P53 fusion proteins. HeLa cells were infected with the above strains at an MOI of 20 for 3 hours. Bacteria were then cleared and cells were collected and lysed. Cell lysates were run on a SDS-PAGE gel and subject to Western blotting with an antibody that detects all 3 isoforms of the Ras.

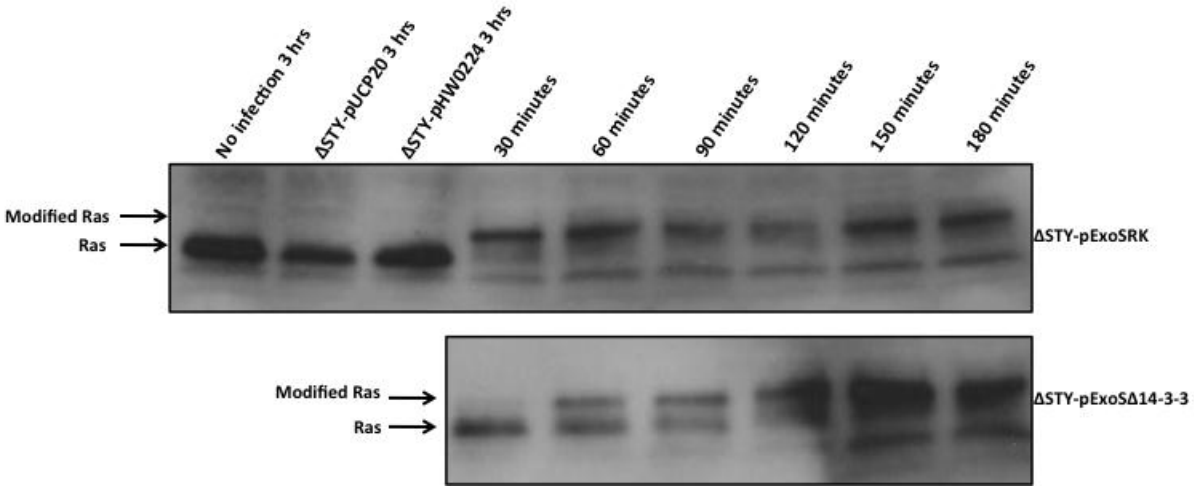

Figure 5-6. Ras modification time course. HeLa cells were infected with either PAK-JΔ*STY*/pExoSRK or PAK-JΔ*STY*/pExoSΔ014-3-3 at an MOI of 20 for various times. At each time point, the bacteria were cleared and the cells were collected and lysed. Cell lysates were subject to Western blotting with an antibody recognizing Ras.

CHAPTER 6
GENERAL DISCUSSION

Summary and Significance of Principal Findings

Delivery of Functional Nuclear Proteins via the Bacterial T3SS

Previous results from our laboratory have demonstrated that our derivate of the *P. aeruginosa* laboratory strain PAK, known as PAK-J, is able to secrete 10 times the amount of effectors as the common strain of PAK (84). We utilized this strain to deliver functional nuclear proteins into eukaryotic cells by fusing the N-terminal 54 amino acids of ExoS to Cre-recombinase. While it has been demonstrated that bacteria possessing a T3SS can be used to deliver proteins into the cytosol of mammalian cells, we are the first to demonstrate the delivery of nuclear proteins (39, 128). Our delivery strain was substantially reduced in cytotoxicity as a result of chromosomally deleting the three T3SS exotoxins (Figure 3-3). Additionally, we demonstrated that the ExoS54-Cre fusion protein was functional by infecting a specialized cell line, which contains the *lacZ* gene blocked by a loxP flanked transcriptional terminator (Figure 3-7A). Our results showed that increasing either the MOI or duration of infection resulted in a greater number of β-galactosidase positive cells resulting from an increase in the amount of injected fusion protein (Figure 3-8). Interestingly, the maximum percentage of β-galactosidase positive cells we were able to achieve with bacterial infection was 45%, whereas infection with a lenti-virus expressing *cre* resulted in almost 100% of the cells staining positive. Results presented in this work demonstrated that the amount of Cre delivered by bacteria was much greater than that produced from the virus however, the lenti-virus continually expresses protein following integration of the vector into the host genome, whereas

bacterial delivered protein is only transient (Figure 3-9). With synchronization of Te26 cells prior to bacterial infection, we were able to increase the amount of β-galactosidase positive cells to 75%, which corresponded to the number of cells that were in S-phase of the cell cycle (Figure 3-12). This suggests that cell cycle plays a role in the recombination efficiency probably resulting from the DNA being more readily accessible to the Cre protein.

The significance of this work is the development of a protein delivery system that could meet some of the current challenges faced in the field of molecular cell biology. Current methods for cellular reprogramming and transdifferentiation of cells rely on the use of integrating viral vectors, which cause reservations about their clinical use (153, 180). The bacterial system is unique in that it can deliver transient amounts of protein that can be altered based on infection time and MOI. Additionally, the bacterial system can be eliminated with use of antibiotics, so there are no lingering effects. Other methods used for cellular reprogramming have tried the use of purified proteins, however this is costly, laborious, and often a tedious procedure (187). The bacterial system solves this issue because the bacteria produce the proteins of interest. While there is still room for improvement in our system, the research presented here is the first step in creating a system that can one day be utilized in a clinical setting.

Discovery of a Novel Cytotoxin

While the strain we generated for protein delivery, PAK-JΔ*STY*, caused few harmful effects to cells during incubations of 4 hours or less, longer incubation times resulted in cytotoxicity (Figure 4-1). In an effort to determine the cause of this toxicity, we identified NDK as a protein that is injected into eukaryotic cells in the absence of the known type III secreted effectors (Figure 4-4). The work presented in this study showed

that NDK is able to elicit a cytotoxic response when expressed in eukaryotic cells, and that this toxicity is independent of the kinase domain (Figure 4-5). While it has been well documented that type I secreted NDK is able to extracellulary elicit a toxic response in macrophages, we are the first to demonstrate toxicity from injected NDK (181).

Our initial findings reaffirmed that NDK is secreted by the T1SS, yet needs a functional T3SS for efficient injection (80). Extracellular complementation assays using two bacterial strains demonstrated that NDK secreted into the extracellular media via the T1SS of a type III defective strain could be injected into cells by the T3SS of a stain lacking NDK (Figure 4-10). Conducting these assays using two type III mutant strains failed to show injection of NDK demonstrating the necessity of the T3SS (Figure 4-11). Additionally, expression of a truncated form of NDK lacking the type I secretion signal failed to be injected into HeLa cells suggesting NDK must be secreted into the extracellular space before it is injected (Figure 4-9).

This study not only identified an additional effector injected via the T3SS of *P. aeruginosa*, but it also serves as evidence in support of newly emerging model for type III secretion in which effectors located outside of the bacteria can be translocated into host cells via the T3SS. This work demonstrates that NDK is localized to the outer membrane of *P. aeruginosa*, suggesting that it binds to the bacteria before it is injected (Figures 4-12, 4-13). Identifying NDK as a novel cytotoxin could prove helpful in the clinical setting as it could be utilized as a target for therapeutic intervention during *P. aeruginosa* infections.

Role of 14-3-3 Proteins in ExoS Activation

One aim of this study was to explore the use of ExoS as an anti-cancer agent. Although bacterial toxins are currently being utilized for cancer treatments, ExoS is a

unique toxin in that it requires activation from a eukaryotic signaling protein in order to generate cytotoxicity (48). We therefore attempted to place ExoS under control of MDM2, a protein that is typically overexpressed in certain forms of cancers, with the goal of generating MDM2 specific killing in cancerous cells. To accomplish this, the 14-3-3 binding domain of ExoS was replaced with variations of the p53 binding sequence for MDM2 (Figure 5-1). Infections assays using cell lines either possessing or lacking MDM2 demonstrated that the ExoS-P53 fusion proteins did elicit a toxic effect however, it was greatly reduced compared to infection from the wild-type form of ExoS and appeared to be independent of MDM2 (Figures 5-3, 5-4). Interestingly, we discovered that these fusion proteins did have functional ADPRT domains, as was evident by their ability to modify Ras proteins (Figure 5-5).

Another noteworthy observation was that a form of ExoS lacking the 14-3-3 binding domain had the ability to modify Ras. It has been well documented that disturbing the 14-3-3 binding domain of ExoS results in reduced cytotoxicity and the inability to ADP-ribosylate Ras, however these studies were carried out using a form of ExoS that is lacking the first 88 amino acids (69). It was later shown that deletion of the MLD, located from amino acids 51-72, prevented the ADP-ribosylation of Ras and reduced cytotoxicity (123). In this study we are the first to demonstrate that a full length form of ExoS lacking the 14-3-3 binding domain, is still able to ADP-ribosylate Ras, although this happens at a much slower rate compared to infection with the wild-type form (Figure 5-6). Perhaps this observation was overlooked due to the fact earlier studies examining the roles of 14-3-3 proteins used truncated forms of ExoS lacking the MLD. Based on these initial results, it appears as though the ADPRT does not need 14-

3-3 for activation, but possibly as a chaperone to facilitate binding to its target proteins. This is based on infection assays that showed ExoSΔ14-3-3 modified Ras at a slower rate than the wild-type form. Taken together, results from this work and previous results suggest that ExoS might need both the MLD and the 14-3-3 binding domain to achieve maximum cytotoxicity.

As a whole, the results presented in chapter 5 showed that forms of ExoS containing the binding domain of p53 for MDM2 were able to elicit toxicity and ADP-ribosylate Ras proteins. Also, this cytotoxicity was reproduced with a form of ExoS lacking the 14-3-3 binding domain. While the mechanism behind 14-3-3 activation of ExoS is still elusive, these results provide new insight into the mechanism explaining ExoS mediated toxicity and pose intriguing questions about the interactions between these proteins.

Future Directions

Bacterial Delivered Proteins

Our initial studies utilizing *P. aeruginosa* for protein delivery demonstrated that we could successfully deliver functional nuclear proteins into mammalian cells. The ultimate goal of this project was to develop a delivery system for cellular reprogramming and transdifferentiation of cells. Since our initial studies, our laboratory has shown that MyoD fused to the first 54 amino acids of ExoS was not only delivered into MEF cells, but that it was functional and able to transform these cells into myocytes. Current work in the laboratory is focused on developing an infection protocol to optimize this process. Work has also begun on generating iPS cells by delivering Oct4 fused to the first 54 amino acids of ExoS into neural progenitor cells, as studies have shown these cells only require the expression of Oct4 for transformation back in to a pluripotent state (85).

Once we have worked out the conditions necessary to accomplish this, we will attempt to deliver the complete set of transcription factors necessary to transform MEF cells into iPS.

Additional research will be conducted to render the delivery strain less toxic. Our current strain lacks the T3SS secreted effectors, and shows minimal toxicity at infection times less than three hours. Our lab has identified NDK as an additional cytotoxin that is injected into host cells via the T3SS. Using the strain PAK-JΔ8, allows for incubation times up to almost 7 hours before toxic effects are seen. Through additional screening, it is possible to identify additional factors that will render our delivery strain less virulent.

Translocation of NDK

Our initial studies suggest that NDK is first secreted into the surrounding medium, and then translocated into cells via the T3SS. Based on evidence from several laboratories, and results presented in this work, it is hypothesized that toxins localize to the bacterial outer surface, and then are injected in a type III dependent fashion. One question that needs to be examined is whether NDK is able to bind to components of the type III apparatus. The most likely candidates would be tip proteins of the translocon such as PopB or PopD however, it is possible that NDK could bind with PscF, the type III needle protein, as was suggested with the *E.coil* protein EspC (167). Binding of NDK to components of the needle complex would support our hypothesis that NDK is bound to the membrane and then pushed into the host cell while the needles are assembling. Another question to resolve is why the human form of NDK is not readily injected. One step to answering that question would be to determine if the human form is located on the outer surface of *P. aeruginosa*. Perhaps it lacks the necessary signal sequence to locate to the outer membrane. It is also possible that it does bind to the outer membrane

but lacks the ability to interact with the needle or translocon. Resolving this question might aid in identifying the signal sequence necessary for type III mediated injection of NDK.

Our results suggest that intracellular NDK is cytotoxic to cells, although the mechanism behind which this occurs is unknown. *P. aeruginosa* stains lacking type III effectors have been implicated in facilitating inflammasome mediated cytotoxicity through activation of caspase-1, so it is possible that NDK is one of the factors responsible (45). Future studies will be conducted to determine if strains lacking NDK are able to stimulate activation of capsae-1. Additionally, studies should be conducted looking at activation of caspase-3 to see if somehow NDK is causing apoptosis.

Finally, it would be interesting to examine if this mechanism of NDK injection is only observed in the PAK-J strain. Previous reports have indicated that another laboratory strain of *P. aeruginosa*, PA01, is unable to secrete NDK. It is possible that there is something different about PAK that allows for NDK secretion. For reasons that still remain unclear, our laboratory has demonstrated that PAK is able to secrete elevated levels of type III effectors compared to PAO1, so perhaps all secretion systems are elevated in PAK. To address this question, complementation studies could be conducted to see if PAK-J secreted NDK could be injected by a functional T3SS from PA01. Alternatively, these same experiments could be conducted with PAK-J and other type III containing bacteria such as *Yersinia* or Shigella to see if the T3SS from other bacterial species is capable of injecting NDK into mammalian cells.

The Role of 14-3-3 Proteins in ExoS Activation

Preliminary results presented in this study showed that ExoS fusions proteins in which the 14-3-3 binding domain had been replaced with p53 binding sequence for

MDM2 were still able to ADP-ribosylate Ras. This same observation was seen with an ExoS protein which had the 14-3-3 binding domain removed. Future experiments will focus on verifying that ExoSΔ14-3-3 is able to modify Ras. Numerous studies have shown that ExoS lacking amino acids 426-431 are unable to bind 14-3-3 proteins and modify Ras, however our construct lacking amino acids 420-453 and does. Future experiments will be performed to confirm that our ExoSΔ14-3-3 is indeed unable to bind 14-3-3 proteins.

To examine if MDM2 is really playing a role in activating the ExoS-P53 constructs, we will employ the use of an in-vitro ADP-ribosylation assay, which is widely used when studying the ADPRT of ExoS. This assay works by combining purified ExoS, Ras, 14-3-3 proteins, and a source of NAD. When 14-3-3 is present, the molecular weight of Ras on an SDS-PAGE will be increased due to modification however, when 14-3-3 is missing from the reaction, Ras remains unmodified. Utilizing this assay with purified MDM2 and the ExoS-P53 fusion proteins should provide insight into whether MDM2 is indeed directly required for activating the ADPRT. This same experiment can also be used to verify that our ExoSΔ14-3-3 does not need 14-3-3 binding for the activation of the ADPRT.

To that end, the first thing that needs to be determined is if 14-3-3 proteins really induce a conformational change in ExoS, or simply act as adaptor proteins for its substrates. If 14-3-3 proteins turn out to act as scaffolds, then perhaps changing the 14-3-3 binding domain will not be helpful for targeting cancer cells. It is possible however, that our ExoSΔ14-3-3 is in a conformation that renders the ADPRT constitutively active, although not as toxic as the wild-type. If this turns out to be the case, then we can focus

on how to make ExoS more responsive to activation from MDM2. This could be tested by moving the p53 binding domains to different areas in the C-terminal portion of ExoS to hopefully identify a form with enhanced responsiveness.

Final Remarks

The work presented in this study demonstrated that we could utilize *P. aeruginosa* as a protein delivery system. It also presented the discovery a novel cytotoxin that requires the cooperation of two different bacterial secretion systems for injection into host cells. However, the exact nature by which this toxin induces cell death and the detailed mechanism explaining injection into the host cell remain elusive. Finally, preliminary results indicated that modifications of the 14-3-3 binding domain of ExoS do not necessarily disrupt the ability of the APDRT to modify proteins, but can alter the severity of the cytotoxic response. Taken together, this study shows how *P. aeruginosa* could be utilized as a tool for cellular reprogramming, the discovery of a novel toxin that is injected via a newly emerging model for type III secretion, and the possibility that 14-3-3 proteins might play a role in ExoS toxicity that was previously unknown.

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BIOGRAPHICAL SKETCH

Dennis Neeld was born in DeLand, Florida in 1984. After completing high school, Dennis enrolled at the University of Florida where he received a Bachelor of Science degree in microbiology and cell sciences in May of 2007. He began his graduate work at the University of Florida in August 2007 in the Interdisciplinary Program in Biomedical Sciences. In 2008 he joined the laboratory of Dr. Shouguang Jin where he studied bacterial pathogenesis caused by the type III secretion system. After completing his PhD in 2012, Dennis plans to pursue a post-doctoral fellowship in immunology related research and eventually one day run his own laboratory.